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ANESTHESIA

XXXIV. CHEMICAL CONSTITUTION OF HYDROCARBONS AND CARDIAC AUTOMATICITY¹

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In a former communication (1) the authors demonstrated that eleven cyclic and acyclic hydrocarbons, when inhaled, sensitized the dog's myocardium to epinephrine. On the other hand, in twelve animals ethylene failed to produce sensitization under varying experimental conditions. Thus it appears that ethylene is unique, in this regard, among the hydrocarbons studied by us and also by Chenoweth (2).

It is known that the aliphatic ethers as a class of anesthetic agents do not produce this type of myocardial sensitization. However, the unsaturated hydrocarbon, ethylene, appears to behave in this respect similar to the aliphatic ethers and dissimilar to the hydrocarbons. It is the purpose of this investigation to pursue further the relationship between chemical constitution and myocardial sensitization to epinephrine.

COMPOUNDS STUDIED. Consideration was given first to inner oxides of hydrocarbons; such molecules as ethylene oxide and propylene oxide have structural properties common to the hydrocarbons and the ethers. Certain rare cyclic hydrocarbons, such as methyl cyclobutane, were included. Spiropentane, owing to its relationship to cyclopropane, was studied. In addition, acetylene was tested to determine whether or not it behaved like its more saturated analog, ethylene.

EXPERIMENTAL PROCEDURE. Electrocardiograms, Lead II, were recorded from the unanesthetized dogs. Epinephrine hydrochloride solution, 1:100,000 (preserved with sodium bisulfite), was injected intravenously, 0.01 mgm./kgm. The injection was made during a period of 25 to 40 seconds. At the end of the injection (usually the beginning of the effect) another tracing was made.

Subsequently each animal was permitted to breathe a mixture of the hydrocarbon in varying concentrations mixed with oxygen. With the liquid hydrocarbons the concentrations were between 10 and 25 per cent. With the gaseous compounds concentrations from 15 to 60 per cent were used. The closed circuit procedure was employed. When the inhalation had continued for from 10 to 20 minutes the foregoing experimental procedures were again carried out.

RESULTS. The various compounds studied and the results observed are shown in table 1.

DISCUSSION OF RESULTS. In the dog, prior to the inhalation of the compound,

¹ The expense of this investigation was defrayed in part by a grant from The Ohio Chemical & Mfg. Co., Cleveland, Ohio.

epinephrine produced slowing of the cardiac rate, accentuation of the T-wave and an occasional inversion of the QRS-complex. Under the influence of the hydrocarbons producing sensitization, however, multifocal ventricular ectopic tachycardia occurred in nearly all experiments. In some animals this passed into ventricular fibrillation and caused death.

From the data in table 1 it will be observed that acetylene was incapable of producing sensitization similar to that produced by saturated hydrocarbons (2). In these dogs, exposure to acetylene² was more comparable to exposure to ethylene than to other hydrocarbons. In four of the twelve animals some sensitization occurred, as evidenced by only a tachycardia with occasional arrhythmia and not ventricular fibrillation. Three of the twelve dogs exposed to acetylene were exposed to cyclopropane on subsequent days. Not any of the three animals showed arrhythmias with epinephrine under acetylene; all three showed multifocal ventricular ectopic tachycardia with epinephrine under cyclopropane.

TABLE 1

NAME OF COMPOUND	NUMBER ANIMALS IN WHICH SENSITIZATION OF MYOCARDIUM OCCURRED AND NUMBER USED
Acetylene.....	4/12
Ethylene (cat).....	0/8
Ethylene Oxide.....	0/2
Propylene Oxide.....	0/2
Spiropentane.....	2/4
Vinyl Chloride.....	3/7
Ethyl Chloride.....	5/6
Isopropenyl Chloride.....	1/3
Methyl Cyclobutane.....	2/2

It appears that among the hydrocarbons, unsaturation reduces the incidence of myocardial sensitization. Thus exposure to ethylene (1) in our experience did not sensitize the dog's myocardium to epinephrine. Nevertheless the introduction of a methyl group into the molecule with the formation of propylene, produced marked cardiac sensitization when inhaled. Similarly, acetylene produced only mild cardiac sensitization in certain animals with epinephrine. In three animals the inhalation of methyl acetylene produced marked arrhythmias and some fibrillation without the injection of epinephrine. By examination of the data in table 1 it is observed further that sensitization with ethyl chloride was produced more often than with vinyl chloride in the eight animals studied.

Garb and Chenoweth (3) conducted their studies on the papillary muscles of the cat's heart. We considered it of interest to study the cat's heart under ethylene anesthesia in order to include another species. Accordingly, we anesthetized eight cats (eleven experiments) with ethylene-oxygen mixtures and subjected them to the experiment. Like the dog, they did not show sensitization.

² "Airco" acetylene was purified by washing with water, concentrated sulfuric acid and 20 per cent sodium hydroxide solution, respectively.

Garb and Chenoweth (3) have suggested the hypothesis that different concentrations of the hydrocarbon reduce myocardial irritability in various portions of the ventricles. This gives rise to small temporary blocks in the presence of certain sympathomimetic amines. Our findings indicate that the changes in myocardial irritability are much less marked with unsaturated hydrocarbons than with saturated compounds. Indeed we have failed to produce the classical effects of sensitization in any of twelve dogs or eight cats with ethylene. However, with acetylene, sensitization of a minor degree was produced in four of twelve dogs when this hydrocarbon was inhaled.

It appears therefore that sensitization of the myocardium is disfavored by double and triple bonds between the carbon atoms in the inhaled hydrocarbon. However, failure to sensitize seems to be a matter of degree and not the absolute absence of some incipient effect. We have not been able to correlate these observations with various differences in the physical properties of saturated and unsaturated hydrocarbons. Nevertheless a distinct diminution in the degree of myocardial sensitization does appear to be associated with the presence of unsaturation in the molecule.

CONCLUSIONS

1. The capacity of acetylene to sensitize the dog's myocardium to epinephrine appears to be intermediate between ethylene and saturated hydrocarbons.
2. Ethylene failed to sensitize the heart of the cat to epinephrine.
3. It appears that vinyl chloride produces sensitization of the myocardium less frequently than does its saturated analog, ethyl chloride.

ACKNOWLEDGEMENT. The authors wish to express their thanks to Dr. Robert H. Oster, of the Department of Physiology of this school, for his assistance in the interpretation of the electrocardiograms.

REFERENCES

1. KRANTZ, J. C., JR., CARR, C. J., AND VITTA, J. F.: *This Journal*, 94: 315, 1948.
2. CHENOWETH, M. B.: *J. Ind. Hyg. and Toxicol.*, 28: 151, 1946.
3. GARB, S., AND CHENOWETH, M. B.: *This Journal*, 94: 12, 1948.

ON THE METABOLISM OF HISTAMINE. A. URINARY
EXCRETION FOLLOWING ORAL ADMINISTRATION.
B. CONJUGATION *IN VITRO*

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A. Urinary excretion following oral administration. On a basis of earlier investigation it had been assumed that little or no histamine is normally excreted in the urine and that administered histamine is almost completely destroyed in the body. However, it was demonstrated by Anrep *et al.* (1) that histamine occurs normally in the urines of various species of animals; the amounts were largely dependent on the dietary composition. It was further shown by them that histamine exists in the urine in two forms: a biologically inactive conjugate that can be converted into free histamine by acid hydrolysis, and free histamine. The urines of *carnivora* contained chiefly the conjugate, while in *herbivora* mainly free histamine was present. Following oral administration of histamine to dogs they recovered from 3 to 5 per cent in the urine, as a conjugate. In an independent investigation, Alexander (2) recovered in the urine an average of 37 per cent of the histamine injected intravenously into mice. Dale and Laidlaw (3) obtained evidence of a biologically inactive derivative in the urine of a cat following subcutaneous injection of histamine.

We have carried out a study of the excretion of histamine in the urine following oral administration to mice, rats, rabbits, guinea pigs, and dogs. The method of determination was a colorimetric procedure applied after preliminary purification of the samples (4). Colorimetric tests were carried out on urines that had been freed of ammonia and subjected to the cotton acid succinate (CAS) method of purification of McIntire, Roth, and Shaw (5). The difference in values before and after acid hydrolysis represented the conjugated histamine. Details of the procedure have been previously described (4).

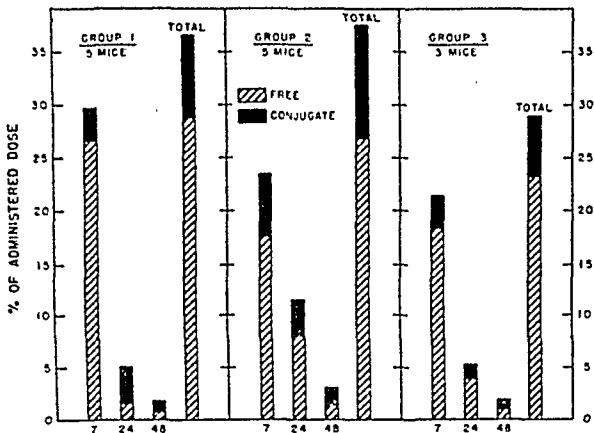
Corollary evidence of the specificity of the method was obtained in several of these experiments by parallel tests with a purified diamine oxidase (histaminase) preparation and, in some urines by bioassay upon the atropinized guinea pig ileum.

Histamine dihydrochloride (synthetic) was used in all experiments. It was administered in neutralized solution through a stomach tube. Urines were collected in metabolism cages, although in rabbits and dogs catheterized specimens were obtained when possible. The diet of rats, mice, and guinea pigs consisted of commercial pellets. Guinea pigs received supplements of cabbage. Rabbits were maintained on cabbage and oats, and dogs on milk and bread. Food was withheld for eighteen hours prior to the experiments. All values are expressed as histamine base.

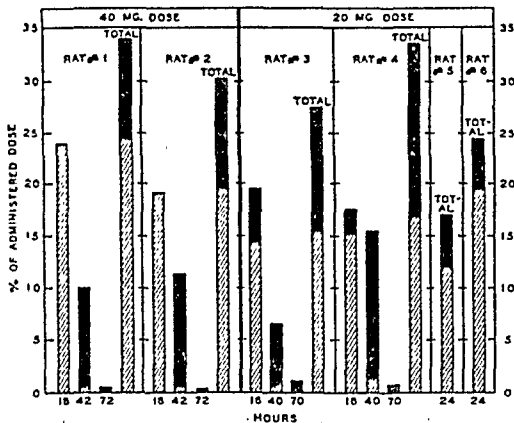
RESULTS. *Mice.* Groups of three to five female albino mice of 18 to 22 gm. weight were given 2 mgm. of histamine base each, in a volume of 0.2 to 0.4 cc. by stomach tube. Two cc. of 0.9 per cent NaCl were injected subcutaneously to promote diuresis. The pooled urines after seven hours contained most of the histamine excreted; an average of 21 per cent of the amount administered was pres-

URINARY EXCRETION OF HISTAMINE

2 MG. ORALLY PER MOUSE

HISTAMINE EXCRETION IN URINE - RATS

FREE (hatched bar) CONJUGATED (solid black bar)



ent as free histamine, and 3.7 per cent as the conjugate. The concentration of free histamine was checked in two 7-hour samples by bioassay. The total excretion in 48 hours averaged 26.3 per cent free and 8 per cent conjugate (fig. 1).

Rats. Single oral doses of 20 or 40 mgm. of histamine were given to albino rats of 180 to 220 gm. weight. Urines were collected in individual metabolism cages.

The total excretion of free and conjugate was of the same order as found in mice; during the first sixteen to eighteen hours, the histamine was largely in the free form, while later samples contained chiefly the conjugate. In four rats the

TABLE 1

The Urinary Excretion of Histamine in Guinea Pigs 20 mgm. histamine base orally to each animal

HOURS AFTER ADMINISTRATION	URINARY EXCRETION		TOTAL EXCRETION PER CENT
	Free	Conjugate mgm.	
7	0	0.27	1.3
24	0	1.46	7.3
48	0	Trace	—
			8.6
7	Trace	0.39	1.95
24	0	0.66	3.3
48	0	0.07	0.35
			—
			5.6
7	0	0.23	1.15
24	0	0.37	1.85
48	0	0	—
			3.0
7	0	0.20	1.0
24	0	0.28	1.4
48	0	0.08	0.4
			—
			2.8

urines at sixteen to eighteen hours contained an average of 18 per cent of the administered dose as free, and 1.7 per cent as conjugate; urines collected at 18 to 72 hours averaged 0.8 per cent as free histamine and 11 per cent as the conjugate (fig. 1).

Guinea pigs. Four guinea pigs of approximately 400 gm. weight were given 20 mgm. histamine orally. The total 48-hour excretion averaged 5 per cent, almost entirely as a conjugate (table 1).

Rabbits. Adult male rabbits were given doses of 40 to 100 mgm. per kgm. of histamine orally. In nine rabbits receiving 100 mgm. per kgm., the 24-hour ex-

TABLE 2
Urinary Excretion of Free and Conjugated Histamine in Rabbits
Following Oral Administration

WT. KGM.	DOSE MG./KGM.	HOURS AFTER ADMIN- ISTRATION	URINARY EXCRETION*		CONCENTRATION IN URINE		PER CENT CONJUGATE
			mgm.	per cent	Free	Conjugate	
					<i>microgram per cc.</i>		
2.25	100	6.5	1.24	0.6	70.0 ¹	53.1	43
		21	4.64	2.1	48.4	67.6	58
		32	0.31	0.1	0	12.3	100
		48	1.0	0.5	0	12.3	100
1.57	100	23	2.50	1.6	4.3	49.0	92
1.84	100	23	2.37	1.3	4.3	26.0	86
2.49	100	5.5	1.01	0.4	66.1 ¹	112.0	63
1.69	75	6.5	0.38	0.3	17.6	37.7	68
		21	2.85	2.2	8.0	21.1	70
		48	2.28	1.8	6.7	35.7	81
1.28	75	7	0.49	0.5	35.3 ¹	62.7	64
2.58	75	6.5	0.81	0.4	154.0 ^{1,2}	197.0	56
1.0	40	6.5	0.22	0.5	2.0 ²	5.0	72
		24	1.31	3.3	0.9	16.3	95
1.23	40	6.5	0.48	1.0	2.7 ²	6.8	72
		21	0.68	1.4	0.5	2.8	85
3.46	100	24	12.6	3.7			
		48	1.39	0.4			
1.87	100	24	5.7	3.0			
		48	0.04	0.02			
1.51	100	24	2.33	1.5			
		48	0.21	0.2			
1.55	100	24	1.67	1.1			
		48	3.59	2.3			
2.28	100	24	10.85	4.7			
		48	0.25	0.1			
2.02	100	24	7.25	3.6			
		48	0.42	0.2			
1.84	75	24	1.22	0.9			
		48	0.11	0.08			

* Non cumulative values for free plus conjugated histamine, excreted between the intervals listed in the third column.

¹ Free histamine (eluted from CAS pad) confirmed by destruction by histaminase.

² Free histamine confirmed by bio-assay (guinea pig ileum).

cretion averaged 2.58 per cent; in seven of them the excretion during the 24- to 48-hour period averaged 0.5 per cent of the administered dose (table 2). During the first 24 hours the conjugated histamine varied from 43 to 100 per cent in the individual experiments. The basis for this variation is not known. A general

TABLE 3
Urinary Histamine Following Oral Administration to Dogs

HISTAMINE ORALLY	HOURS AFTER ADMINISTRATION	URINARY EXCRETION		TOTAL EXCRETION PER CENT
		free	total	
mgm.		mgm.	mgm.	
282*	control	—**	—	
	5 hours	—	trace	
	21	0.097	4.0	1.4
	29	0.40	6.75	2.4
	48		5.25	1.9
				5.7
391*	control	—	—	
	5	—	trace	
	21	0.241	4.87	1.25
	29	0.336	4.62	1.18
	48		5.2	1.33
				3.76
200	control		—	
	6½		1.0	0.5
	24	trace	22.5	11.2
	32		12.0	6.3
	48		3.6	1.8
				19.8
200	control		—	
	6½	.067	1.8	0.9
	24	.080	13.3	6.6
	32		1.37	0.68
	48		1.07	0.53
				8.71

* 30 mgm. per kgm. of histamine base. ** — Signifies negative.

parallelism was observed between the percentage of free histamine excretion and the manifestations of toxicity.

In the period from 24 to 48 hours, from 84 to 100 per cent of the histamine in the urine of rabbits was present as a conjugate.

Further evidence of the validity of the results obtained with the colorimetric test upon rabbit urine was furnished by a few experiments with diamine oxidase

(histaminase) and by bioassay upon the atropinized guinea pig ileum. The histaminase studies were carried out with a highly purified enzyme preparation (6). Tests were made both upon diluted urines and upon the neutralized acid eluates from the CAS pads. Satisfactory agreement in the results with the different methods was obtained.

Dogs. Following the oral administration of 36 to 180 mgm. of histamine base to dogs, Anrep and coworkers (1) recovered from 3 to 5 per cent in the urine, as a conjugate.

The results of four experiments we have conducted upon dogs are shown in table 3. Because of the occurrence of conjugated histamine in the urine of dogs on a meat diet (1), they were kept on milk and bread for several days prior to the experiment. It was also found necessary to morphinize the dogs (10 mgm. per kgm., subcutaneously) before the administration of histamine, to prevent vomiting.

From 3.76 to 19.8 per cent of the administered dose (200 to 391 mgm.) appeared in the urine. From traces to 7 per cent of the amounts present in individual samples was estimated as free histamine, on a basis of its behavior towards the CAS pads.

Toxicity of orally administered histamine. Toxic manifestations have been observed in all of the species studied. While it is known that histamine introduced into the alimentary canal can be absorbed, and that lowering of blood pressure and stimulation of gastric secretion can be produced, no real toxicity by this route had been established (7).

Recently Parrot, Gabe, and Herrault (8) reported an LD_{50} of approximately 0.165 gm. per kgm. orally for guinea pigs, with 6 per cent mortality at 0.06 gm. per kgm. The lesions produced were characteristic of histamine intoxication, plus ulceration, and in some instances, perforation of the gastric mucosa.

Our results on guinea pigs are in agreement with those of Parrot and coworkers. We have also carried out toxicity studies on rabbits and mice with results shown in table 4. The LD_{50} for rabbits was approximately 0.1 gm. per kgm. Eight of thirteen rabbits that succumbed to 0.1 gm. per kgm. showed evidence of gastric perforation at autopsy. The values for mice were obtained from several experiments performed over a period of one year, and the irregular results indicate variations in toxicity that we have been unable to correlate with season or preliminary fasting. Female mice from one source were used. It is of interest that the oral toxicity for mice and rats is much closer to the parenteral toxicity than it is in the more susceptible species studied.

Guinea pigs and mice sacrificed within 30 minutes after oral histamine showed considerable congestion of the stomach, small intestines, kidneys and spleen. After several hours the congestion was decreased but much fluid was present in the intestinal canal.

Mice and rats are relatively resistant to histamine. By intravenous injection they can tolerate from 200 to 1000 times as much as guinea pigs and rabbits. The demonstration that a large percentage of the histamine administered to mice and rats is excreted in the urine unchanged is evidence that their tolerance is not

based upon conjugation or destruction of histamine. In fact the results in this study show a much more active destruction of histamine in those species sensitive to histamine than in mice and rats.

B. The conjugation of histamine by liver preparations in vitro. Following the demonstration by Anrep and coworkers (1) that histamine can occur in the urine in the form of a biologically inactive conjugate, attempts have been made to establish the nature and site of conjugation. These authors suggested the intestine or liver as the probable site.

TABLE 4

Toxicity of Orally Administered Histamine for Rabbits and Mice. Values Expressed as Histamine Base; 4 to 8 per cent Solutions Neutralized Before Administration

NUMBER OF RABBITS	DOSE GM. PER KGM.	NUMBER OF DEATHS	PER CENT MORTALITY
3	0.040	1	33
10	0.075	2	20
26	0.100	14	54
<hr/>			
NUMBER OF MICE			
40	0.100	5	12.5
20	0.200	4	20.
30	0.400	1	3.3
30	0.800	4	13.3
20	1.600	10	50.
<hr/>			
MICE INTRAPERITONEAL INJECTION*			
10	0.2	0	0
10	0.4	0	0
10	0.8	7	70.

* These experiments were carried out simultaneously with those in which an LD_{50} of approximately 1.6 gm. per kgm. was obtained by oral administration.

The chemical behavior of the conjugate suggested that it might be acetyl histamine, 4(β -acetyl aminoethyl) imidazole (4). This compound was later isolated in crystalline form in appreciable amounts from the urine of dogs, following the administration of histamine orally, by Tabor and Mosettig (9). Urbach (10) likewise demonstrated acetyl histamine in the urine of man and the dog, by the use of paper chromatography. He also observed acetylation upon the addition of histamine to the large intestinal contents and stools, and suggested the large intestine as a site of conjugation. The available evidence indicates that acetyl histamine is the principal, if not the only, conjugation product.

Preliminary studies in the rabbit following oral administration of histamine have shown the presence of the conjugate in the liver and bile (11). Likewise the conjugate has been demonstrated in the urine following the subcutaneous administration of histamine to normal rats and to rats with the entire intestinal canal below the stomach removed (11). These results indicate that conjugation can occur outside the alimentary canal, and experiments described below demonstrate that liver preparations *in vitro* are capable of conjugating histamine.

Methods. Free and conjugated histamine were determined by a colorimetric method previously described (4). When applied to trichloroacetic acid extracts of tissues, it was necessary to remove the trichloroacetic acid before the extract could be put through the cotton acid succinate (CAS) pad for purification.

This tedious step has been avoided by the use of metaphosphoric acid as a protein precipitant, since sodium metaphosphate in the amounts required is not extracted by butanol in quantities sufficient to interfere with the CAS pads. Sulfuric acid extracts of tissues can also be used for the determination of free histamine by this method (12), but they are less satisfactory for total histamine because of incomplete protein precipitation.

The weighed tissue is ground with acid washed sand in a mortar, with one volume of 25 per cent metaphosphoric acid and three volumes of water. After standing 20 minutes it is filtered.

For the determination of free histamine, 4 cc. of the filtrate are cooled in ice and neutralized (spot test with phenolphthalein) with 5 *N* sodium hydroxide (approximately 0.4 cc.). To this are added 1.4 gm. of the salt mixture (5), and the solution is extracted with 5 cc. of butanol. The butanol extract is passed through a CAS pad, which is later washed with 1 cc. of 0.014 *M* trisodium phosphate to remove any conjugated histamine; free histamine is eluted from the pad with acid, and the colorimetric test applied to the eluate. The details have been previously described (4).

Conjugated histamine is determined as the increase in free histamine which occurs upon acid hydrolysis. Four cc. of the metaphosphoric acid filtrate are hydrolyzed in a test tube with 0.2 cc. of 10 *N* sulfuric acid for three hours in boiling water, with occasional addition of water to prevent evaporation to dryness. The hydrolyzed extract is washed into a graduate with approximately 2 cc. of water, cooled in ice and approximately 1.1 cc. of 5 *N* sodium hydroxide slowly added to bring the pH of the solution to 11.5 to 12 (a spot test with indigo disulfonate begins to turn green). The final volume is made up to 4.5 cc. The salt mixture and butanol are now added, and aeration carried out if considerable ammonia is present.

Rabbit or pigeon livers are removed immediately upon exsanguination. Thin slices or minced tissues of approximately 1 gm. weight are placed in small flasks containing 4 cc. of Krebs' Ringer-bicarbonate solution or Ringer-phosphate buffer (13) of pH 7.4. Sodium acetate is added to give a final concentration approximately 0.01 molar. Following histamine addition the flasks are either incubated at 37°C. with a slow stream of 95 per cent O₂ and 5 per cent CO₂ bubbling through, or those with phosphate buffer are shaken in a water bath at 37°C.

In the fresh liver preparations the two processes of (a) conjugation and (b) destruction of histamine by histaminase proceed simultaneously under aerobic conditions. In our experiments, 1 gm. of fresh pigeon liver destroyed from 13 to 90 microgm. of histamine in the course of five hours.

If a small amount of histamine is added destruction may take place before appreciable conjugation has occurred. If a large excess is used the technical difficulty is encountered of determining small quantities of conjugate in the presence of large amounts of free histamine. To obviate this difficulty, where a large excess of histamine is present, the flasks are incubated with a purified diamine oxidase (histaminase) preparation (6) for an additional one or two hours at the end of the experiment. In this way most of the free histamine is destroyed while the conjugate is not attacked. At the end of this period the metaphosphoric acid extracts are prepared and analyzed for free and conjugated histamine.

Several experiments with rabbit liver slices showed conjugation of added histamine, but the activity was variable. Since the process was believed to be an acetylation, and since pigeon liver has been found to be a favorable source for the

acetylation of sulfanilamide (14, 15), experiments were repeated upon pigeon liver preparations; more consistent results were obtained, with from 10 to 20 microgm. of histamine conjugated per gm. of wet tissue. The results of three experiments with fresh liver preparations are shown in table 5.

TABLE 5

In Vitro Conjugation of Histamine by Liver Preparations

All values are expressed on basis of microgm. per gm. of fresh tissue

ANIMAL LIVER PREPARATION	MICROGRAM OF HISTAMINE BASE ADDED	TIME HRS.	MICROGM. OF HISTAMINE CONJU- GATE FOUND	PERCENT CONJU- GATED
Rabbit, slices.....	22	5	4.7	21.8
Pigeon, slices.....	129	6	19.4	15
Pigeon, minced.....	50	4.5	10.5	21.5

TABLE 6

Conjugation by "Aged" Extracts of an Acetone Powder from Pigeon Liver

AMOUNT OF POWDER	HISTAMINE ADDED, MICROGRAM	PERIOD OF INCUBATION, HOURS	FREE HISTAMINE, MICROGRAM	TOTAL HISTAMINE, MICROGRAM	PER CENT CONJUGATED
100 mgm.....	50	3	46	46	—
100 mgm.*.....	50	3	23	26	—
100 mgm. + 15 units CoA.....	50	3	23	43	40
100 mgm. + 15 units CoA*.....	50	3	Trace	17.7	35
200 mgm. powder†.....	50	4	36	36	—
200 mgm. + 15 units CoA.....	50	4	Trace	30	60
200 mgm. + 15 units CoA.....	50	4	Trace	36	72
100 mgm.*.....	50	1	11.4	12.1	—
100 mgm. + 11 units CoA*.....	50	1	10.	23.2	26

* Histaminase added at the termination of the experiment.

† 100 mgm. at start, 100 mgm. after 2 hours.

The medium employed is described by Kaplan and Lipmann (17), and contains adenosine triphosphate, acetate, citrate, and cysteine. A volume of 1 cc. was used in the first experiment and 2 cc. in the lower two. Incubation was carried out in small test tubes at 37° C. in air.

Advantage was taken of the fundamental studies of Lipmann and coworkers (14, 16, 17) upon the enzyme system involved in certain biological acetylations. Employing the acetylation of sulfanilamide as a model, they have obtained an active enzyme preparation from an acetone precipitate of pigeon liver, and have isolated, and partially identified chemically, coenzyme A, which is required in the reaction. Acetylation proceeds in the presence of adenosine triphosphate, sodium acetate, and cysteine.

We have carried out studies upon the conjugation of histamine, employing an acetone powder of pigeon liver and a coenzyme A preparation (potency of 15

units per mgm.) kindly supplied by Drs. Lipmann and Novelli, as well as similar preparations made by us. The conditions employed were those described by Kaplan and Lipmann (17) for the assay of coenzyme A. The results of three such experiments are shown in table 6. Similarly to the acetylation of sulfanilamide, the conjugation of histamine proceeded only in the presence of coenzyme A. It remains to be determined whether enzymatic deacetylation (15) also takes place under the conditions of these experiments. This evidence, along with the recovery of acetyl histamine from the urine (9, 10), suggests that the nature of the reaction is an acetylation. It has not been established whether the enzyme involved in the conjugation of histamine is the same as that which acetylates sulfanilamide.

The histaminase activity observed in fresh pigeon liver slices was largely absent in the acetone powder obtained from Drs. Lipmann and Novelli, and in two similar powders which we have prepared.

SUMMARY

The excretion of free and conjugated histamine in the urine has been determined after oral administration to mice, rats, rabbits, guinea pigs, and dogs. In mice and rats up to 37 per cent of the administered dose could be recovered largely in the free form. In the other species from 3 to 20 per cent was recovered chiefly as conjugated histamine.

Rabbit and pigeon liver slices *in vitro* were capable of conjugation of histamine. Conjugation was also obtained with a cell free extract prepared from an acetone powder of pigeon liver; with this preparation it was shown that coenzyme A is required for the process.

REFERENCES

1. ANREP, G. V., AYADI, M. S., BARSOUM, G. S., SMITH, J. R., AND TALAAT, M. M.: *J. Physiol.*, 103: 155, 1944.
2. ALEXANDER, F.: *Quart. J. Exper. Physiol.*, 33: 71, 1944.
3. DALE, H. H., AND LAIDLAW, P. P.: *J. Physiol.*, 41: 319, 1910.
4. ROSENTHAL, S. M., AND TABOR, H.: *THIS JOURNAL*, 92: 425, 1948.
5. MCINTIRE, F. C., ROTH, L. W., AND SHAW, J. L.: *J. Biol. Chem.*, 170: 537, 1947.
6. TABOR, H.: To be published.
7. GADDUM, J. H.: *Gefasserweiternde Stoffe der Gewebe*. Georg Thieme, Leipzig, 1930.
8. PARROT, J. L., GABE, M., AND HERRAULT, A.: *Compt. rend. Soc. de Biol.*, 141: 484, 1947.
9. TABOR, H., AND MOSLETTIG, E.: *J. Biol. Chem.*, (In press).
10. URBACH, K. F.: *Proc. Soc. Exper. Biol. and Med.*, 70: 146, 1949.
11. MILLICAN, R. C.: Unpublished data.
12. ROSENTHAL, S. M.: *J. Natl. Cancer Inst.*, (In press).
13. KREBS, H. A., AND HENSELEIT, K.: *Zeit. f. Physiol. Chem.*, 210: 33, 1932; 217: 191, 1933.
14. LIPMANN, F.: *J. Biol. Chem.*, 160: 173, 1945.
15. KREBS, H. A., SYKES, W. A., AND BARTLEY, W. C.: *Biochem. J.*, 41: 622, 1947.
16. NOVELLI, G. D., AND LIPMANN, F.: *J. Biol. Chem.*, 171: 833, 1947.
17. KAPLAN, N. O., AND LIPMANN, F.: *J. Biol. Chem.*, 174: 37, 1948.

THE BRONCHODILATOR ACTIVITY OF SOME ANALOGS OF N-ISOPROPYLARTERENOL (ISUPREL)¹

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Since Konzett's (1) disclosure of the high bronchodilator activity of 1-(3',4'-dihydroxyphenyl)-2-isopropylaminoethanol (Isuprel), various investigations (2-4) have been carried out with this drug and its analogs in an effort to determine the structural requirements for this action. Corrigan, Langermann and Moore (5) have prepared a series of N-substituted analogs of 1-(3',4'-dihydroxyphenyl)-2-aminoethanol and these were made available to our laboratory for pharmacological evaluation. The results obtained are described here and represent an extension of an investigation of the effects on bronchodilator and cardiovascular action (2, 3) of varying the N-alkyl group.

METHOD. The method for producing experimental asthma in guinea pigs herein described is a modification of the technics employed by Schaumann (6), v. Issekutz (7) and Loew (8). Briefly, it consists of blowing into a glass chamber containing the guinea pig a histamine mist produced by an ordinary commercial nebulizer using a 0.2 per cent solution of histamine diphosphate. The technic has been described in detail in a previous publication (2). Results are recorded in minutes under two columns designated ONSET and DURATION.

RESULTS. The results of the histamine aerosol tests are reported in table 1. The comparative doses listed are based upon the amount of drug necessary to increase the reaction time approximately 100 per cent over the control time obtained from untreated guinea pigs.

The N-isopropyl drug (Isuprel) caused the greatest amount of bronchodilatation, showing two and one-half times the potency of the N-n-butyl analog, the next most active bronchodilator drug by this test. Increase in the size of the N-alkyl group appears to decrease the bronchodilator effect, as with the n-amyl (WIN 5596), while branching of the side chain appears to contribute markedly to activity, since the isopropyl analog shows ten times the activity of the N-n-propyl (WIN 5587) derivative. Both the cyclopentyl and cyclohexyl analogs (WIN 5591 and WIN 5589) were found to be comparatively weak bronchodilator drugs, but were much more active than branched N-alkyl derivatives larger than isopropyl, namely the 3"-amylamino (WIN 5592) and the isobutyl (WIN 5595) compounds. These latter drugs show the marked diminution in effect which characterizes compounds with large branched-chain alkyl substituents.

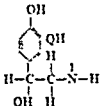
The drugs were also tested by the lung perfusion method of Sollman and von Oettingen as modified by Thornton (9). The results are summarized in table 2.

* Isuprel is the registered trade mark of Winthrop-Stearns, Inc. for racemic 1-(3',4'-dihydroxyphenyl)-2-isopropyl-aminoethanol hydrochloride.

WIN 5590, the n-butyl analog of Isuprel, is one of the most active bronchodilators in the intact animal, but shows relatively poor action in the perfused lung. The activity of the cyclopentyl analog of Isuprel was surprising inasmuch as it was slightly more potent than Isuprel (ratio of 0.41) in the perfused lung, whereas in the intact animal this substance was found to be less potent than Isuprel, both by simultaneous aerosolization (table 3) and by the histamine asthma

TABLE 1

Bronchodilator action of eight analogs of Isuprel against bronchoconstriction in guinea pigs induced by histamine aerosols

	STRUCTURE	NO. OF EXPTS.	DOSE	CONTROL		EXPERIMENTAL†		INCREASE OVER CONTROL TIME	APPROX. ISUPREL RATIO**	TOXICITY
				Onset of symptoms	Duration of exposure	Onset of symptoms	Duration of exposure			
Compound WIN	I		mgm / kgm.	min.	min.	min.	min.	per cent*		(l.v. mouse) approx. LD ₅₀
Isuprel	isopropyl	20	0.01	0.72	1.13	1.47	2.63	118	1	83
5557	n-propyl	7	0.10	0.48	0.81	1.08	1.68	137	10	95
5590	n-butyl	7	0.025	0.46	0.73	1.10	1.40	115	2.5	85
5596	n-amyl	14	0.10	0.61	1.12	1.70	2.52	145	10	36
5593	n-methyl isobutyl	7	0.10	0.55	0.91	0.96	1.46	67	15-20	67
5591	cyclopentyl	14	0.10	0.46	0.82	0.76	1.28	64	15-20	53
5589	cyclohexyl	13	0.10	0.67	1.30	1.45	2.05	87	12	67
5592	3* amyl-amino	7	0.50	0.60	1.05	1.02	1.01	77	80	75
5595	isobutyl	7	0.25	0.45	0.80	0.90	1.40	57	43	60

* Per cent increase represents the average increase over control for onset and duration times observed after administration of the test compound.

** Approx. Isuprel ratio = mgm. test drug causing 100 per cent time increase/mgm. Isuprel causing 100 per cent time increase; all values expressed in terms of the base.

† ONSET equalled the time necessary to produce prominent symptoms, namely, the increase in respiratory frequency, forced inspiration, etc. DURATION represents the total time from exposure until asphyxial convulsions or collapse were produced.

tests as described elsewhere (2). There appears to be no readily apparent explanation for this variance.

The most active derivatives in this group were compared directly with Isuprel by aerosolizing the bronchodilator drug rather than by intraperitoneal injection. The concentration of the test drug necessary to provide approximately equal increases over the control times was used as the basis for comparison. Both histamine and the test drug were placed in the nebulizer and aerosolized simultaneously. The results are shown in table 3. The differences in the effectiveness ratios obtained in these tests are much more marked than in the experiments

TABLE 2

Bronchodilator action of eight analogs of Isuprel against histamine-induced constriction in perfused guinea pig lungs

COMPOUND	NUMBER EXPERIMENTS	BRONCHODILATOR DOSE (MICROGM. OF BASE)	RESPONSE TO HISTAMINE*	RESPONSE TO HISTAMINE + DILATOR	ISUPREL RATIO**
			cc./min.	cc./min.	
Isuprel	6	1.0	53/19	50/57	1.0
	6	2.5	64/30	57/66	
5591	6	0.43	72/32	65/59	0.41
	6	0.65	56/22	53/58	
5589	6	3.9	68/33	61/46	5.2
	6	7.7	67/32	61/66	
	6	39	51/24	50/56	
5593	6	4.0	64/32	62/53	4.4
	6	8.0	51/17	48/59	
5592	6	4.0	50/27	49/41	4.5
	6	8.0	50/21	46/53	
5595	6	8.6	66/33	60/45	14.4
	6	21.5	63/31	61/70	
	6	43.0	55/25	42/55	
5587	6	3.9	57/26	55/50	3.6
	6	5.8	53/21	49/59	
	6	7.8	57/25	54/62	
5590	8	4.3	55/24	51/41	6.0
	9	8.6	64/29	55/58	
5596	8	2.1	62/29	56/49	2.5
	8	4.3	51/15	43/48	

* Constriction induced by injecting 10-30 microgm. of histamine acid phosphate directly into the perfusion cannula just above the lungs.

** Expressed as multiples of the effective dose (ED100) of Isuprel. All doses expressed in terms of the base.

TABLE 3

Comparative antihistaminic activity of two analogs of Isuprel when aerosolized with histamine

COMPOUND WIN	NO. EXP.	DOSE* (DILUTION)	CONTROL†		EXPTL†		INCREASE PER CENT**	APPROX. ISUPREL RATIO††
			Onset of symptoms	Duration of exposure	Onset of symptoms	Duration of exposure		
Isuprel	19	1:2,000,000	0.55	0.96	1.63	2.33	165	1
5590	6	1:50,000	0.68	1.06	1.75	2.50	147	40
5591	8	1:35,000	0.45	0.86	1.10	1.90	132	60

* Dose expressed in terms of the base.

** Average per cent increase of both onset and duration times.

† See footnote to table 1.

†† Expressed as multiples of the effective dose of Isuprel. All doses expressed in terms of the base.

wherein the drugs were injected intraperitoneally, where the dosage is precisely controlled. Both the cyclopentyl and the n-butyl derivatives were found to be distinctly less active. The cyclopentyl analog shows about two-thirds as much activity as the n-butyl by the simultaneous aerosolization test, whereas in the histamine asthma test it has only one-tenth as much bronchodilator effect.

Toxicity results are shown in table I. Results are expressed as the approximate LD_{50} and were determined by intravenous injection into albino mice. The animals were housed under conditions of constant humidity and temperature. The toxicity of Isuprel was found to be comparable to that of drugs in the series possessing low Isuprel ratios, but these differences are small. These drugs generally have a large therapeutic ratio.

Discussion. While Isuprel is, perhaps, still the most potent bronchodilator substance, with its structure fulfilling most nearly the apparently specific requirements for bronchodilation, it still has certain undesirable side-effects (Lands, 3). Since Konzett (1) and others (2-4) have demonstrated that the catechol nucleus and the presence of an alcoholic hydroxyl on the beta carbon of the side chain are important for effective bronchodilation, variation in structure in this series was made only by varying the substituent on the amino nitrogen. In a previous investigation (2) it was suggested that the straight chain N-alkyl derivatives were only slightly active. This finding is in agreement with the results we have obtained with the n-propyl and n-amyl analogs of Isuprel. However, the n-butyl derivative shows a very high activity in the intact guinea pig, although this is not apparent in experiments on the perfused lung (see tables 1 and 2). This may indicate that the n-butyl group undergoes some change in the intact animal that renders it more potent, while in the isolated lung this does not occur. Certain drugs may also have their pharmacologic effects diminished when applied directly to the musculature of the bronchi, as witness the difference in effect of the n-butyl analog in the intact animal when given intraperitoneally, when injected into the perfusion system of the isolated lung, or aerosolized directly into the lungs of intact animals. This latter technique yields results agreeing better with those obtained with the isolated perfused lung. This is not surprising, since the two techniques are essentially the same. It should not be necessary to point out that in evaluating these data the results obtained with the intact animal are perhaps most significant inasmuch as the conditions of the experiment most nearly approximates those in actual clinical bronchoconstriction. The information obtained in this investigation does not seriously conflict with the idea expressed in the previous investigation (2) wherein it is suggested that the N-isopropyl radical represents the optimal group activating the receptor mechanism in the bronchi. It would appear, however, that both the n-butyl (WIN 5590) and the cyclopentyl (WIN 5591) analogs of Isuprel possess good bronchodilator activity. A further investigation of these compounds to determine their clinical efficiency and side-effects seems warranted.

CONCLUSIONS

1. The bronchodilator effects of eight analogs of Isuprel (1-(3',4'-dihydroxyphenyl)-2-isopropylaminoethanol) are described.

2. While Isuprel remains the most potent bronchodilator drug in the intact animal, the n-butyl and cyclopentyl analogs possess marked activity.

3. The cyclopentyl analog possesses a bronchodilator activity equal to or exceeding that of Isuprel in the perfused lung but has only one-twentieth of the activity in the histamine-asthma test. Some possible explanations are suggested and discussed.

ACKNOWLEDGMENT. The authors wish to express their gratitude to Dr. J. O. Hoppe and Mr. D. Seppelin for the toxicity determinations described in this communication.

REFERENCES

1. KONZETT, H.: Arch. f. exper. Path. u. Pharmacol., 197: 27, 1940.
2. SIEGMUND, O. H., GRANGER, H. R., AND LANDS, A. M.: THIS JOURNAL, 90: 254, 1947.
3. LANDS, A. M., NASH, V. L., DERTINGER, B. L., GRANGER, H. R., AND MCCARTHY, H. M.: THIS JOURNAL, 92: 369, 1948.
4. MARSH, D. F., PELLETIER, M. H., AND ROSS, V. A.: THIS JOURNAL, 92: 108, 1948.
5. CORRIGAN, J. R., LANGERMANN, M., AND MOORE, M. L.: J. Am. Chem. Soc., 71: 530, 1949.
6. SCHAUMANN, O.: Arch. f. exper. Path. u. Pharmacol., 196: 109, 1940.
7. v. ISSEKUTZ, B., AND GENERSICH, P.: Arch. f. exper. Path. u. Pharmacol., 202: 201, 1943.
8. LOEW, E., KAISER, M., AND MOORE, V.: THIS JOURNAL, 83: 120, 1945.
9. THORNTON, J. W.: Quart. J. Exper. Physiol., 21: 305, 1932.

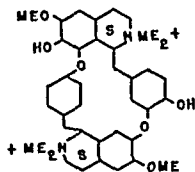
THE CURARIFORM ACTIVITY OF N-METHYLBERBAMINE AND N-METHYLISOTETRANDINE¹

DAVID F. MARSH AND D. A. HERRING

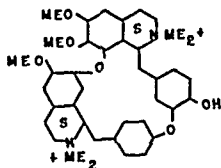
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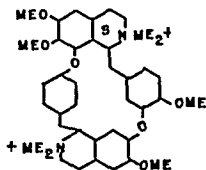
Of the known biscoclaurine type alkaloids, berbamine (1) and its O-methyl ether, isotetrandine (2) are found in a variety of plants.² We have converted



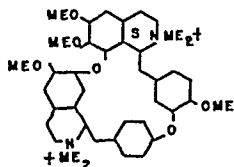
D-TUBOCURARINE



N-METHYLBERBAMINE



D-O-METHYLTUBOCURARINE



N-METHYLISOTETRANDINE

d-Tubocurarine: Used as the dichloride-pentahydrate, molecular weight 785.7; m. p. 268-269°C.

d-O-Methyltubocurarine: Used as the diiodide-trihydrate, molecular weight 909.7; m. p. 257-258°C.

N-Methylberbamine: Used as the diiodide, molecular weight 892.6; m. p. 258-261°C.

N-Methylisotetrandine: Used as the diiodide, molecular weight 906.6; m. p. 265-268°C.

these agents to their quaternary ammonium derivatives, the methiodides, and compared their pharmacological activity with the spatially related d-tubocurarine chloride and d-O-methyltubocurarine iodide.

¹ Part of the material in this paper was presented before the American Society for Pharmacology and Experimental Therapeutics, Federation meetings, Detroit, 1949. See *Fed. Proc.*, 8: 318, 1949.

² This research was supported in part by a grant from S. B. Penick & Co., New York. We are grateful to Dr. W. G. Bywater, S. B. Penick & Co., New York, for crude extracts of

EXPERIMENTAL PROCEDURE. Head drop assays in 24 rabbits, three trained unanesthetized dogs and one man, and toxicity studies in 120 rats were carried out by the methods employed in an earlier investigation (3).

Cats. Two hundred fifty mgm. of sodium barbital per kgm. were administered intraperitoneally 60 minutes prior to operation in nine cats (2.1-2.9 kgm.). The femoral and sciatic nerves to one leg were cut. The peripheral end of the cut sciatic nerve was stimulated

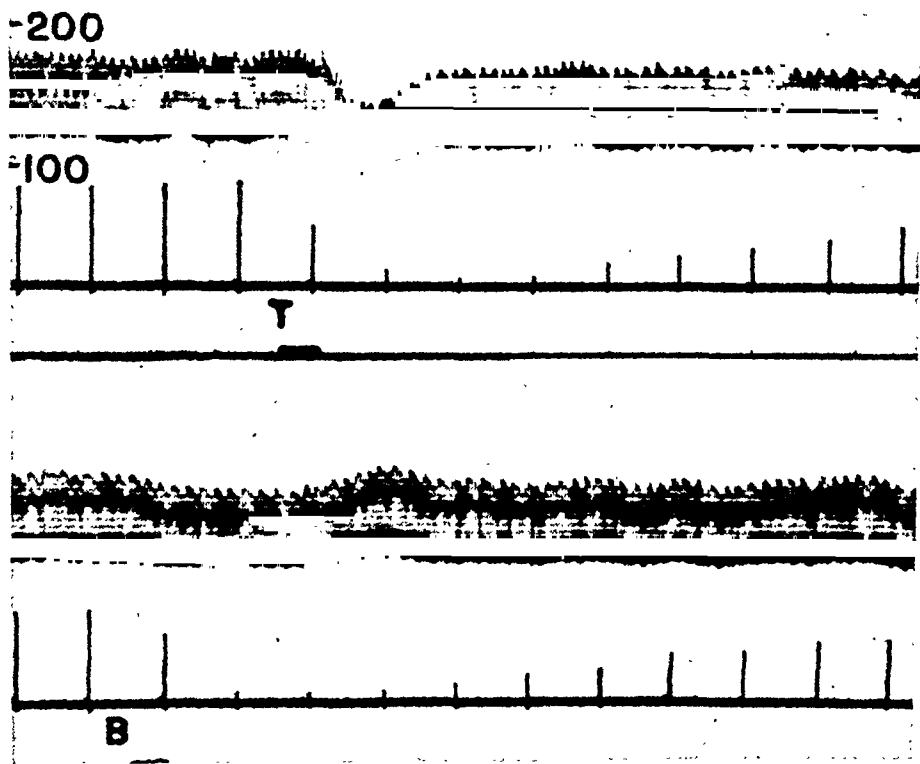


FIG. 1. Cat (2.1 kgm.). Blood pressure, in mm. Hg, above, and gastrocnemius contractions, below. Peripheral end of cut sciatic nerve stimulated every 10 seconds. Eighteen minutes deleted between portions of record. One hundred twenty-five microgm. d-tubocurarine chloride pentahydrate per kgm. given at T; 150 microgm. N-methylberbamine iodide per kgm. given at B.

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lever. The blood pressure in the opposite femoral artery was simultaneously recorded with a Lambert-Wood (4) strain gage manometer. The rabbit head drop dose of a curariform agent was administered intravenously and after the muscle had returned to normal, 0.125 mgm. of d-tubocurarine chloride pentahydrate per kgm. was given. If the two effects were not equivalent, the dose of the first agent was increased or decreased as necessary until an effect equal (within $\pm 5\%$) to that produced by the d-tubocurarine was obtained. The equivalent doses given in table 1 are based on three cats for each compound. No more than four injections were given to any animal and no injections were made less than fifteen minutes apart. See fig. 1 for portions of a typical record.

In an additional eight barbitalized cats the cardiovascular response to 2 microgm. of epinephrine hydrochloride, acetylcholine chloride, and histamine acid phosphate per kgm. was determined with a Lambert-Wood strain gage in the femoral artery. The equivalent paralytic dose determined above was administered, and the epinephrine, acetylcholine, and histamine injections repeated. Following this either atropine sulfate (0.69 mgm./kgm.) or diphenhydramine hydrochloride (2.9 mgm./kgm.) was given and the curariform agent readministered periodically every five minutes until respiratory collapse occurred.

Isolated Tissue Segments. Hearts from four rabbits were prepared for perfusion by the Langendorff technique and varying amounts of the agents added to the Ringer-Locke perfusion fluid. Isolated sections of ileum from two rabbits were prepared for recording by the usual Magnus technique in Tyrode solution and the responses to acetylcholine chloride and to histamine phosphate were tested before and after addition of the curariform agents. The tracheas of four guinea pigs were prepared by the method of Castillo and de Beer (5). Ten microgm. of curariform agent were added to each cc. of bath. The largest increase in tone from this concentration of d-tubocurarine chloride was equivalent to that produced by 0.02 microgm. histamine base per cc. Further experiments were carried out to determine the ability of this concentration of the agents to antagonize the contractile effect of 0.5 microgm. of acetylcholine chloride per cc.

RESULTS. The results are summarized in table 1. It is not difficult to determine the curariform activity of these highly specifically acting agents. It is difficult to determine with any accuracy the secondary or side-effects that these agents have. It may be noted that the relative effect on skeletal muscle does vary among species and among the various methods of assay although the relative order of the compounds remains constant.

d-Tubocurarine chloride usually has little action in unanesthetized man other than that related directly to depression of skeletal muscular function (6), although such diverse side-effects as throbbing headache, bronchoconstriction, cold sweat, vertigo, fall in blood pressure, salivation, and flushing of the extremities have been reported (7). Landmesser (8) has established that this agent produces bronchoconstriction in dogs and some similar effect has been observed in man (9). Unanesthetized dogs that have had paralytic doses of this agent exhibit marked salivation, and often vomiting, lacrimation, and urination (10). No such side-effects are observed in rats or rabbits.

As indicated in table 1, the d-tubocurarine and its methyl ether always produce some fall in blood pressure in the cat even at comparatively low doses. This fall in blood pressure is not prevented by the administration of atropine or diphenhydramine. In general, these compounds have very little effect on the response to epinephrine, acetylcholine or histamine, although occasional slight increase in effect by epinephrine and acetylcholine is observed. In comparison; N-methylberbamine and its methyl ether have much less effect on blood pressure

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THE CHEMICAL BASIS FOR ADRENERGIC BLOCKING ACTIVITY IN COMPOUNDS RELATED TO DIBENAMINE^{1, 2, 3}

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The obvious experimental and therapeutic usefulness of a relatively nontoxic drug specifically blocking the effects of epinephrine and sympathetic nerve activity has stimulated considerable pharmacological research designed to develop such an agent. An entirely new series of adrenergic blocking agents, the β -haloalkylamines, of which N,N-dibenzyl- β -chloroethylamine (Dibenamine) is the prototype, has recently been reported (1-4) to act quite specifically to block certain excitatory responses to epinephrine and other sympathomimetic amines and to sympathetic nerve stimulation.

The present report deals with certain chemical and pharmacological properties of 113 compounds most of which are related chemically to Dibenamine. These have been selected as illustrative examples of a larger series of 218 compounds studied in the same manner. A preliminary report of part of this work was presented in 1946 (5). Since that time studies on a few members of this series in addition to Dibenamine have been published by other investigators (6-8). Several active compounds were reported inactive (7) because of inadequate testing methods. Most of the compounds studied by others are members or minor variations of the basic series presented below, and in all cases their potentialities for producing adrenergic blockade are qualitatively predictable on the basis of the requirements for activity revealed in the present study. The details of synthesis and chemical characterization of these compounds will be presented elsewhere.

METHODS. Stock solutions of quaternary compounds and of the hydrochloride or hydrobromide salts of secondary and tertiary amines were prepared in acidified propylene glycol, as described for Dibenamine (2), and diluted 1 to 10 with 0.9 per cent NaCl solution immediately before injection. Compounds listed as insoluble formed a fine precipitate when so diluted, but the resulting suspension was usually suitable for injection without

¹ ED. NOTE: This paper represents a portion of the work for which Dr. Nickerson was awarded the annual John J. Abel Prize in Pharmacology for 1949 by the Society for Pharmacology and Experimental Therapeutics.

² We wish to express our appreciation to Dr. J. Nikawitz and Mr. G. M. Nomaguchi for assistance with the chemical and pharmacological aspects of this work. The senior author is also indebted to Dr. George V. Beard, Department of Chemistry, University of Utah, and Dr. Linus Pauling, California Institute of Technology, Pasadena, California, for helpful discussion of the reported structure-activity relationship.

³ N,N-dibenzyl- β -chloroethylamine is now being distributed for investigational use by the Smith, Kline and French Laboratories, Philadelphia, Pa., under their trademark, Dibenamine.

error in dosage. When compared on a molar basis the salts and the free bases were found to have essentially the same potency.

Adrenergic blocking activity was determined in cats anesthetized with pentobarbital or urethane and prepared for direct carotid pressure recording. Actual reversal of the blood pressure response to epinephrine was selected as the criterion of activity because mere reduction of the pressor response may result from a number of non-specific factors. In most preliminary tests, 10 or 15 mgm./kgm. doses of the experimental drug were injected slowly intravenously and the blocking action tested at intervals by the intravenous injection of 2.5 microgm./kgm. of epinephrine. If no reversal of the vasopressor response to epinephrine occurred within one-half to one hour after the first injection, the initial dose was repeated at intervals over a period of four to six hours until the limit of tolerance of the animal was reached. In acute experiments this was usually 45 to 100 mgm./kgm., and it was sometimes much larger than the LD_{50} given in the tables. After reversal of the epinephrine response was obtained, the completeness of the blockade was checked with larger doses of epinephrine (50 to 500 microgm./kgm.). The persistence of the blockade was then followed for several hours by injecting small test doses of epinephrine. Compounds which appeared to be inactive by the above method were further tested by injecting the maximal tolerated dose subcutaneously two or three times a day for three to four days and then testing the blood pressure response to epinephrine.

Compounds with a delayed onset of action were rechecked for rate of onset by a standard procedure of injecting 10 mgm./kgm. (occasionally more with compounds of low activity) intravenously over a period of five minutes and then testing the response to 2.5 microgm./kgm. of epinephrine at intervals. When more exact determinations of potency were desired for comparative purposes the drug was administered intravenously to cats anesthetized with pentobarbital and the adrenergic blockade checked one-half and 1½ hours later with 1 microgm./kgm. epinephrine; if the response to this dose was reversed, larger amounts of epinephrine were employed to determine the completeness of the blockade. This procedure minimized the complications which arise because of the fact that epinephrine tends to inhibit the adrenergic blocking action during the period of its development. The foregoing experiments were designed to determine the *minimal effective dose* of the compounds tested.

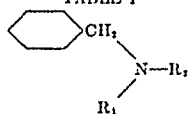
Toxicity was tested by subcutaneous injection of the compounds into mice; all deaths within a ten-day period after injection were recorded. Because of the limited number of animals used in testing each compound (10 to 30, the larger number being employed where considerable variations in response were encountered), the toxicity figures can be considered only as approximate. The experiments extended over a period of four years and recheck experiments indicated that seasonal and strain variations in the mice caused 20 to 40 per cent variations in the estimated LD_{50} of some compounds. Intravenous and intraperitoneal toxicity studies were not undertaken for reasons discussed elsewhere (2).

Solubility was recorded in terms of the maximum pH at which the compound is soluble in aqueous solution to the extent of 0.02 *M*. This test of solubility was selected as being simple to perform and probably parallel to the solubility of the drug in body fluids. Compounds not soluble to the extent of 0.02 *M* at pH 0 are listed as insoluble, although the activity of some of these "insoluble" compounds indicates significant solubility in body fluids.

RESULTS⁴. In order to simplify a comparison of various chemical groupings, substitutions in each of the three positions of the amine will be considered separately. Series A (table 1) includes all substitutions for the β -chloroethyl group in a molecule which is otherwise active. Series B (table 2) includes compounds which retain the β -chloroethyl group and one benzyl group. Series C (table 3) includes compounds retaining the β -chloroethyl group, but having substitutions

⁴ Compounds A-5, C-43 and D-1 were supplied through the courtesy of Drs. J. E. Kerwin and E. J. Fellows, Smith, Kline and French Laboratories, Philadelphia, Pa.


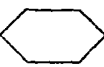

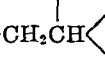


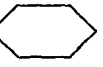



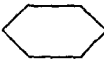
TABLE 1



No.	R ₁	R ₂	SOLUBIL- ITY*	APPROX. LD ₅₀ * mgm./ kgm.	ACTIV- ITY*
Diben- amine	CH ₂ —	—CH ₂ CH ₂ Cl	2.7	800	++
A-1	CH ₂ —	—CH ₂ CH ₂ Br	Insol.	500	++
A-2	CH ₂ —	—CH ₂ CH ₂ CN	Insol.	850	—
A-3	CH ₂ —	—CH ₂ CH ₂ CH ₂ Cl	2.6	850	—
A-4	CH ₂ —	$\begin{array}{c} \text{CH}_3 \\ \\ \text{—CH}_2\text{CHCl} \end{array}$	2.0	500	++
A-5	CH ₂ —	$\begin{array}{c} \text{CH}_3 \\ \\ \text{—C—CH}_2\text{Cl} \\ \\ \text{CH}_3 \end{array}$	Insol.	1000	++
A-6	CH ₂ —	$\begin{array}{c} \text{Cl} \\ \\ \text{—CH}_2\text{C} \begin{array}{c} \diagup \text{cyclohexane ring} \diagdown \end{array} \\ \\ \text{H} \end{array}$	Insol.	>1000	+
A-7	CH ₂ —	$\begin{array}{c} \text{Br} \\ \\ \text{—CH}_2\text{C}=\text{CH}_2 \end{array}$	1.0	1000	—
A-8	CH ₃ —	$\begin{array}{c} \text{Br} \\ \\ \text{—CH}_2\text{C}=\text{CH}_2 \end{array}$	5.7	1000	—
A-9	CH ₂ —	—CH=CH	Insol.	500	—
A-10	CH ₂ —	—CH ₃	5.0	75	—

* See text for explanation.

TABLE 1—Continued

NO.	R ₁	R ₂	SOLUBIL- ITY*	APPROX. LD ₅₀ *	ACTIV- ITY*
				mgm./ kgm.	
A-11	 CH ₂ —	—CH ₂ CH ₂ OH	5.7	1000	—
A-12	CH ₃ CH ₂ —	—CH ₂ CH ₂ OH	>7.0	750	—
A-13	HOCH ₂ CH ₂ —	—CH ₂ CH ₂ OH	>7.0	>1000	—
A-14	 CH ₂ —	$\begin{array}{c} \text{CH}_3 \\ \\ \text{—CH}_2\text{CHOH} \end{array}$	4.2	1000	—
A-15	 CH ₂ —	$\begin{array}{c} \text{OH} \\ \\ \text{—CH}_2\text{CH} \end{array}$ 	Insol.	1000	—
A-16	 CH ₂ —	—CH ₂ COOH	>7.0	600	—
A-17	 CH ₂ —	—CH ₂ CH ₂ —O—CH ₂ CH ₃	4.1	>1000	—
A-18	CH ₃ CH ₂ —	—CH ₂ CH ₂ —O— 	5.1	1000	—
A-19	 CH ₂ —	—CH ₂ CH ₂ —O— $\begin{array}{c} \text{O} \\ \\ \text{C} \end{array}$ 	Insol.	600	—
A-20	 CH ₂ —	$\begin{array}{c} \text{CH}_2\text{—CH}_2 \\ \diagup \quad \diagdown \\ \text{—CH}_2\text{CH}_2\text{N} \quad \text{O} \\ \diagdown \quad \diagup \\ \text{CH}_2\text{—CH}_2 \end{array}$	5.3	450	—
A-21	 CH ₂ —	$\begin{array}{c} \text{CH}_2\text{CH}_3 \\ \diagup \\ \text{—CH}_2\text{CH}_2\text{N} \\ \diagdown \\ \text{CH}_2\text{CH}_3 \end{array}$	4.8	100	—

on or for both of the benzyl groups. A number of compounds which do not fall into any of these categories are grouped in table 4. Dibenamine is included in all of the tables as a basis for comparison.

The compounds have been listed as active or inactive. Those compounds which are markedly more or less active than Dibenamine are designated as (+++)

and (+), respectively. A few special potency comparisons are presented in the text. In most cases there was a clear distinction between active and inactive compounds, and only a very few active agents were found to differ from Dibenamine by a potency factor of five or more. Certain members of the Dibenamine series appear to be significantly more potent than the parent compound. The potency relationships among these highly active compounds have been examined in detail and will be reported elsewhere.

Except in cases where dosage is severely limited by the toxicity of the compound, the procedures employed are capable of detecting an activity approximately 3 per cent that of Dibenamine.

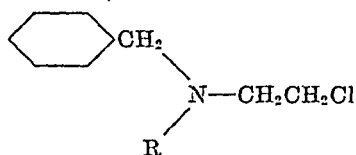
Series A. The compounds shown in table 1 clearly indicate that a halogen attached to the beta carbon of the aliphatic chain is essential to activity. Alkyl (A-10) and various alcohol (A-11 to A-15), carboxyl (A-16), ether (A-17, A-18), ester (A-19) and other non-halogen substitutions in this position lead to complete inactivation, as also does transfer of the halogen to the gamma carbon (A-3). Even the chemically similar —CN radical causes inactivation (A-2), presumably because it lacks the high reactivity of the alkyl halides. Other substitutions on the aliphatic chain, such as the β -methyl of compound A-4 and the β -phenyl of compound A-6, appear to have only minor effects on activity. Double substitution on the β -carbon causes no significant reduction in activity (A-5). Unfortunately, uncontrolled rearrangements have to date prevented the synthesis of the α -disubstituted isomer of A-5. A α - γ unsaturation completely abolishes activity (A-7, A-8).

The presence of two or three β -chloroethyl radicals is characteristic of the nitrogen mustard "gases" of which compounds C-3 and C-4 are examples. The β -chloroethyl radical represents the link between the Dibenamine series of adrenergic blocking agents and the nitrogen mustards. Active compound B-11 may be considered as a true nitrogen mustard.

Series B. The compounds shown in table 2 all possess one benzyl and one β -chloroethyl group, and illustrate two important points regarding the structural requirements for activity in this series: the necessity for the tertiary amine structure and the wide variety of substituents compatible with activity as long as one β -haloalkyl and one benzyl group are present. After substitution of certain other active groupings (e.g., fluorenyl, C-43) for the benzyl, the nature of the third substituent on the amine appears to have a greater effect on activity (8). All secondary amines are inactive (B-1, C-46). Among the other members of this group, the only substitutions leading to inactivation are those (B-2, B-3) containing a second tertiary amine.

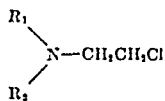
Substitutions ranging from methyl (B-4) to lauryl (B-10) in the aliphatic series, and including such diverse groupings as β -chloroethyl (B-11), allyl (B-12), methylcarbityl (B-13), β -phenoxyethyl (B-19 to B-23), β -pyridylethyl (B-26), β -1-benzotriazolethyl (B-27), thenyl (B-28), etc. have only moderate effects upon the activity. When allowances are made for differences in solubility, it appears that the substitution of an aliphatic grouping for one of the benzyl groups in Dibenamine causes a significant, but not great reduction in activity.



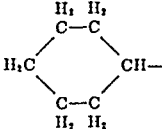
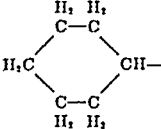
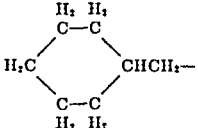







TABLE 2



NO.	R	SOLUBILITY*	APPROX. LD ₅₀ * mgm./kgm.	ACTIVITY*
Diben-amine		2.7	800	++
B-1	H—	>7.0	500	—
B-2		5.0	500	—
B-3		Insol.	500	—
B-4	CH ₃ —	6.4	50	+
B-5	CH ₃ CH ₂ —	5.8	75	++
B-6	CH ₃ CH ₂ CH ₂ —	4.6	100	++
B-7		4.6	100	++
B-8	CH ₃ CH ₂ CH ₂ CH ₂ —	4.1	125	++
B-9	C ₇ H ₁₅ —	Insol.	225	++
B-10	C ₁₂ H ₂₅ —	Insol.	250	++
B-11	ClCH ₂ CH ₂ —	3.0	50	++
B-12	CH ₂ =CHCH ₂ —	4.2	100	++
B-13	CH ₃ O(CH ₂) ₂ OCH ₂ CH ₂ —	5.6	50	++
B-14	CH ₃ OCH ₂ —	Insol.	300	++
B-15	CH ₂ —	1.4	>1000	++

TABLE 3



NO.	R ₁	R ₂	SOLU- BILITY*	APPROX. LD ₅₀ * mgm./ kgm.	ACTIV- ITY*
Diben- amine	 CH ₂ —	 CH ₂ —	2.7	800	++
C-1	CH ₃ —	CH ₃ —	>7.0	250	—
C-2	CH ₃ CH ₂ —	CH ₃ CH ₂ —	>7.0	125	—
C-3	CH ₃ CH ₂ —	ClCH ₂ CH ₂ —	>7.0	<1.0	—
C-4	CH ₃ CH ₂ CH ₂ CH ₂ —	ClCH ₂ CH ₂ —	4.6	1.0	—
C-5	C ₆ H ₁₃ —	CH ₃ CH ₂ —	5.6	75	—
C-6			5.0	20	—
C-7		CH ₃ CH ₂ —	5.6	75	—
C-8		CH ₃ CH ₂ —	0.6	400	+ Very Slight
C-9	 CH ₂ CH ₂ —	 CH ₂ CH ₂ —	Insol.	150	+ Very Slight
C-10	 CH ₂ CH ₂ —	CH ₃ CH ₂ —	5.7	40	+
C-11	 CH ₂ CH ₂ CH ₂ —	 CH ₂ CH ₂ CH ₂ —	Insol.	65	—
C-12	 CH ₂ CH ₂ CH ₂ —	CH ₃ CH ₂ —	5.7	40	—

*See text for explanation.

TABLE 3—Continued



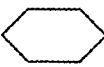
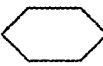
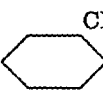
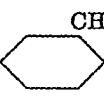
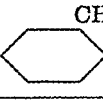
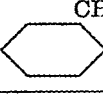
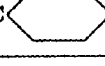
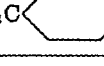
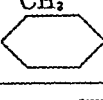
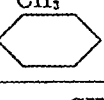
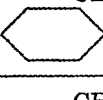
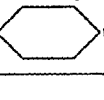
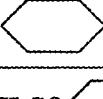
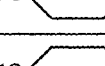
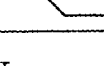
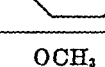
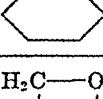
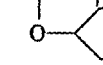
NO.	R ₁	R ₂	SOLU- BILITY*	APPROX. LD ₅₀ * mgm./ kgm.	ACTIV- ITY*
C-13	 CH=CHCH ₂ —	CH ₃ CH ₂ —	5.8	50	++
C-14	 —O—CH ₂ CH ₂ —	 —O—CH ₂ CH ₂ —	Insol.	35	+
C-15	 —O—CH ₂ CH ₂ —	CH ₃ CH ₂ —	5.3	40	+
C-16	 —O—CH ₂ CH ₂ —	 —O—CH ₂ CH ₂ —	Insol.	>1000	+++
C-17	 —O—CH ₂ CH ₂ —	CH ₃ CH ₂ —	4.0	40	++
C-18	 —S—CH ₂ CH ₂ —	CH ₃ CH ₂ —	5.0	80	+ Weak.
C-19	H ₃ C  CH ₂ —	H ₃ C  CH ₂ —	Insol.	1000	++
C-20	 CH ₂ —	 CH ₂ —	Insol.	1000	++
C-21	 CH ₂ —	 CH ₂ —	Insol.	1000	+ Slow Onset.
C-22	 CH ₂ —	CH ₃ CH ₂ —	4.8	100	++
C-23	H ₃ CO  CH ₂ —	H ₃ CO  CH ₂ —	Insol.	300	++
C-24	H ₃ CO  CH ₂ —	CH ₃ —	5.4	50	+
C-25	 CH ₂ —	CH ₃ —	6.5	150	++
C-26	 CH ₂ —	CH ₃ CH ₂ —	5.5	100	+

TABLE 3—Continued

No.	R_1	R_2	SOLU- BILITY*	APPROX. LD ₅₀ * mg./ kgm.	ACTIV- ITY*
C-27			Insol.	>1000	—
C-28			Insol.	>1000	—
C-29			Insol.	>1000	—
C-30		CH_3CH_2-	4.5	100	+
C-31			Insol.	>1000	+ Very Slight
C-32		CH_3CH_2-	3 S	200	+++
C-33	CH_3CH_2-	CH_3CH_2-	Insol.	>1000	—
C-34	CH_3CH_2-	CH_3CH_2-	4.5	25	—
C-35			Insol.	>1000	—
C-36		CH_3CH_2-	4.2	50	—
C-37		CH_3CH_2-	Insol.	1000	—
C-38		CH_3CH_2-	4.9	125	++

TABLE 3—Concluded

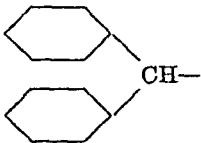
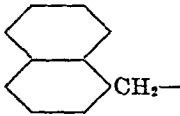
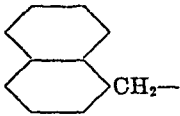
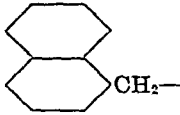
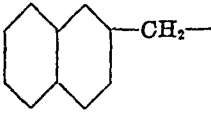
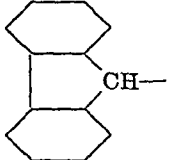
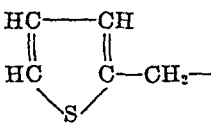
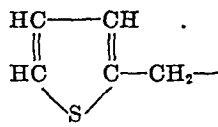
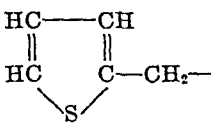
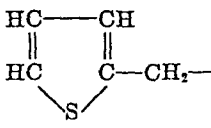
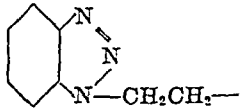
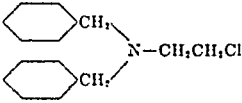
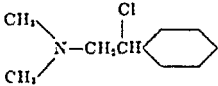
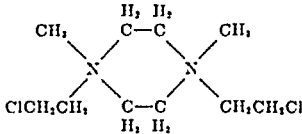
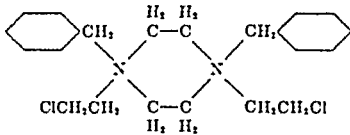
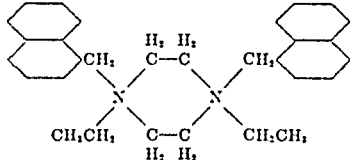
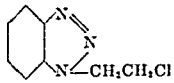
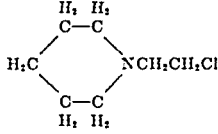
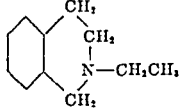
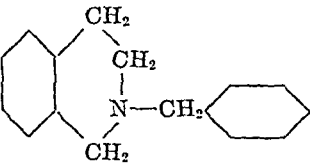
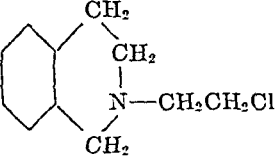
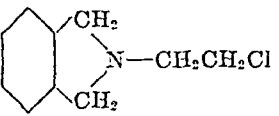
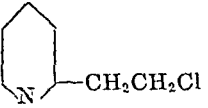
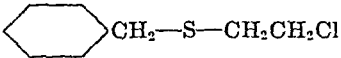
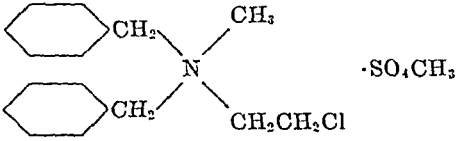
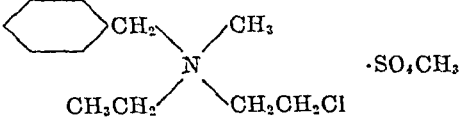
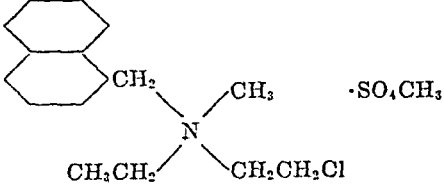
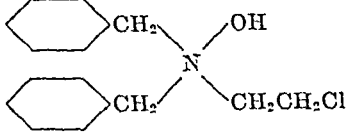
NO.	R ₁	R ₂	SOLU- BILITY*	APPROX. LD ₅₀ * mgm./ kgm.	ACTIV- ITY*
C-39		CH ₃ CH ₂ —	2.3	500	++
C-40			Insol.	>1000	—
C-41		CH ₃ CH ₂ —	3.9	100	+++
C-42		CH ₃ CH ₂ —	3.8	60	±
C-43		CH ₃ CH ₂ —	1.9	800	+++
C-44			1.4	1000	+
C-45		CH ₃ CH ₂ —	5.0	25	+
C-46		H—	>7.0	200	—
C-47		CH ₃ CH ₂ —	>7.0	500	++

TABLE 4

NO	STRUCTURE	SOLU- BILITY*	APPROX. LD ₅₀ * mgm./ kgm.	ACTIVITY*
Diben- amine		2.7	800	++
D-1		>7.0	25	++
D-2		>7.0	1000	-
D-3		>7.0	350	-
D-4		—	500	-
D-5		Insol.	>1000	-
D-6		>7.0	125	-
D-7		>7.0	250	+ Short du- ration

* See text for explanation.

TABLE 4—Continued

NO.	STRUCTURE	SOLU- BILITY*	APPROX. LD ₅₀ * mgm./ kgm.	ACTIVITY*
D-8		5.0	500	
D-9		6.0	100	+ Weak and short du- ration.
D-10		6.9	150	—
D-11		4.1	500	—
D-12		Insol.	— —	—
D-13		5.2	100	++
D-14		7.0	65	++
D-15		4.0	400	++
D-16		Insol.	200	+

cation with large doses. The interposition of a single carbon atom yields compounds which have full activity; for example Dibenamine. The presence of two carbon atoms between the aromatic ring and the nitrogen again causes almost complete inactivation; compound C-9 has about the same activity as C-8. However, further lengthening to the cinnamyl (C-13) or phenoxyethyl (C-14 to C-17) produces very active compounds. The marked activity of C-13 as compared to the inactivity of C-11 and C-12 is of special interest and will be discussed in detail below. In all combinations, the phenylthioethyl substitution (B-24, C-18) produces compounds with a weaker adrenergic blocking action than the corresponding phenoxyethyl.

A final group of compounds in this series is that in which the phenyl ring has been replaced by another aromatic or by an unsaturated heterocyclic radical. Compounds in which an unsaturated cyclic nucleus is separated from the nitrogen by a methylene group are active as long as the previously mentioned activity requirements of other substituents are met (C-41 to C-45). Compound C-42 represents an exception to the general activity of this group. The reason for this is not completely clear. It is possible that both chemical and steric factors are involved in the difference in activity of the α - and β -naphthyl derivatives. Here, as in the case of compounds containing a benzyl group (B-1), secondary amines are completely inactive (C-46). Inasmuch as the substitution of two saturated radicals uniformly leads to inactivity (C-1 to C-7), compounds with saturated heterocyclic substitutions were not prepared.

A few compounds which do not fit into any of the three categories previously discussed are also of interest (table 4). Several compounds in this series (D-2, D-3, D-5, D-6, D-9, D-10) contain a β -chloroethylamine in which the nitrogen is attached to the same ring structure by two bonds. These compounds uniformly lack Dibenamine-type activity. Compounds D-7 and D-9 possess adrenergic blocking activity with quite different properties than those characteristic of the Dibenamine series. Compounds D-9 and D-10 may be considered as derivatives of active compounds B-5 and B-4, respectively, in which the N-methyl and N-ethyl radicals have been attached to the benzene ring in the two positions. Compounds D-13 to D-15 are quaternary derivatives of active compounds; all of those listed are active although slight reductions in activity are noted when these are compared quantitatively with the corresponding tertiary amines. Part, but not all, of the reduced activity may be due to the fact that these compounds were obtained in the form of resins with a maximum purity of 90 per cent. The N-oxide D-16 is significantly less active than the quaternary salts listed.

DISCUSSION. The active adrenergic blocking agents of the β -haloalkylamine (Dibenamine) series show a high degree of specificity both in their pharmacological activity (2-4) and in the chemical configuration necessary for such activity. The blocking action is limited to the inhibition of certain excitatory effects of epinephrine, related amines and sympathetic nerve impulses; inhibitory effects are essentially unaltered. These blocking agents also appear not to affect the release of epinephrine or sympathin or the penetration of these exciting agents

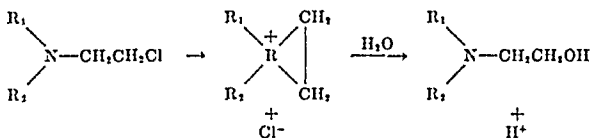
into effector cells. The response (e.g., contraction of smooth muscle) to other effective stimuli is not reduced. Present information indicates that the β -haloalkylamine blocking agents, when carefully administered have very little physiological effect in the animal body except to alter for a rather prolonged period some process or substance necessary for the excitatory response of specific effector cells to sympathomimetic agents. Their action is, therefore, highly selective. The results reported above indicate that the chemical structure required for the production of selective adrenergic blockade by members of the Dibenamine series is also quite specific. It is perhaps not surprising that the high degree of pharmacological specificity exhibited by these agents should be dependent upon an equally specific chemical configuration.

In evaluating the influence of structure on pharmacological activity it is important to consider the reactions by which drugs may produce their effects. Most of the previously studied organic drugs are not very reactive from a chemical point of view and it is usually assumed that they are approximated to their sites of action by relatively weak (van der Waals or hydrogen bond) forces. Consequently, investigations of structure-activity relationship within series of pharmacologically active agents usually have been focused upon steric factors. Alterations in the gross shape and size of the molecule and the distances between "prosthetic groupings" (9) are the usual factors considered. The Dibenamine-type compounds, however, are essentially alkylating agents capable of stable bonding to various tissue constituents, and consequently their chemical properties are of decisive importance in determining blocking activity.

During the early stages of development of the Dibenamine adrenergic blockade, an equilibrium between epinephrine and the blocking agent is readily demonstrated and the presence of sympathomimetic agents markedly inhibits the production of the blockade. This type of "equilibrium" or "competitive" blockade is characteristic of all other types of blocking agents studied in detail (10-12). However, once the Dibenamine blockade has become established it is essentially complete and cannot be overcome by massive doses of the exciting agent. These features of the blockade were first demonstrated on the blood pressure responses of anesthetized animals (1, 2) and on the cat nictitating membrane (3), and have since been confirmed by studies on blood pressure responses of pithed cats and on the isolated rabbit uterus (13). The Dibenamine blockade has also been shown to develop slowly, not reaching its maximum until at least $1\frac{1}{2}$ hours after intravenous administration. Other members of the series vary widely in their rate of action and in some cases (C-21) blockade may not appear for several hours. Other unusual features of the Dibenamine-type blockade are its long duration (2, 4, 6, 14) and the fact that the blockade apparently persists for many hours after effective amounts of active drug have disappeared from the body (4).

All the above characteristics of the Dibenamine adrenergic blockade can be best explained on the basis of a firm bonding of the drug to some tissue constituent with the resultant destruction or prolonged inactivation of some substance in the cell which is essential for excitation by adrenergic stimuli. Fortunately, known reactions of β -chloroethylamines provide a reasonable basis for such an action.

In slightly alkaline aqueous solution β -chloroethylamines have been shown to undergo the following reactions (15, 16).



Dimers, such as compound D-3 (formed from B-11) and D-4 (from C-41) are also formed under these conditions (15).

In studies on the chemistry of the sulfur mustards, which are capable of many of the same reactions as the nitrogen mustards, it has also been demonstrated that in the case of sulfonium (17) and sulfone (18) derivatives, a direct unsaturation of the β -chloroethyl with formation of a vinyl group may occur. These vinyl compounds undergo essentially the same reactions as the sulfide mustards. It is not clear whether a similar vinylamine is formed on splitting of the ethyleniminium ring of the nitrogen mustards, or as an intermediate in the reaction of active quaternary derivatives of the nitrogen mustards (19) and members of the Dibenamine series of blocking agents (D-13 to D-16).

In vitro studies with the nitrogen mustards clearly demonstrate that these compounds may react with sulfhydryl, amino, imidazole and carboxyl groups and other constituents of biological systems in aqueous solution at physiological pH (20, 21). In all cases the observed reactions appear to require formation of intermediates of the type discussed above (20).

It is well known (see 17, 18, 22) that thiosulfate reacts rapidly and competitively with both immonium and vinyl intermediates formed from nitrogen or sulfur mustards, but not directly with the β -chloroethyl groups of the parent compounds. It is therefore of interest that prior administration of thiosulfate will prevent the typical blocking action of Dibenamine and its congeners, including quaternary derivatives; this indicates that these agents do not produce their blocking effect before the formation of intermediates. Inasmuch as the final hydrolysis products, such as A-11 to A-15, are uniformly inactive and all the dimers (D-2 to D-4) which have been studied are also completely inactive, it may be concluded that the substances immediately responsible for adrenergic blocking activity are intermediate transformation products. The rather slow production of the active intermediates in the body may also explain the slow onset of action which is observed even after intravenous administration of Dibenamine and many of its congeners.

All the β -chloroethyl amines which have been studied in detail appear to react in a qualitatively similar manner with tissue constituents, thiosulfate, etc. However, the data presented above clearly point to the fact that relatively minor alterations of other substituents of the amine may drastically affect adrenergic blocking activity. The most probable interpretation of these observations is that the specific blocking activity is dependent upon a narrow range of chemical

properties of the intermediate products formed in the body. Data are available to show that in closely related compounds other substituents on the amine may markedly affect the reactions of a β -chloroethyl group. It has been shown that formation of a β -hydroxyethyl group on ethyl-bis(β -chloroethyl)amine and other nitrogen mustards causes a much decreased rate of ethylenimmonium hydrolysis (16). It is also known that the substitution of ethyl or larger radicals for the methyl groups of dimethyl- γ -bromopropylamine alters its characteristic reaction in the presence of alkali from a linear polymerization to an internal cyclization analogous to that discussed above for the β -chloroethylamines (23). Under the proper circumstances such alterations in reactivity might well lead to qualitatively different pharmacological actions; *i.e.*, activity or inactivity.

On the basis of the above discussion of the mechanism of the adrenergic blocking activity of members of the Dibenamine series, certain tentative generalizations may be made regarding the structural requirements for activity. It appears that an active compound must fulfill the following four requirements:

1. It must be a tertiary amine or the quaternary derivative of an active amine.
2. It must include at least one β -haloalkyl group capable of forming an intermediate ethylenimmonium (or vinyl) derivative with loss of the halogen.
3. It must include an unsaturated ring structure attached to the nitrogen in such a way as to allow resonance stabilization of the active intermediate. A corollary is that any substitution on the ring which interferes with this stabilizing action will lead to decreased activity or inactivity depending upon the magnitude of the effect.
4. It must not have any substitutions on the aromatic ring of the benzyl group which tend to be out of the plane of the ring. This requirement does not apply to substitutions on phenoxyethyl derivatives.

We have observed no exceptions to the first requirement. All the secondary amines tested in this series were found to be completely inactive. This is probably closely related to the second requirement. The highly reactive ethylenimmonium compounds which are known to be intermediates in the action of the nitrogen mustards and which are probably involved in the activity of this series of compounds, are all quaternary derivatives of tertiary amines. The analogous sulfide derivatives such as D-12 are also completely inactive.

The β -haloalkyl grouping may be considered as the primary reacting portion of the molecule. It is essential to activity (series A). It is not surprising that the γ -chloropropyl compound is inactive because it would form a much more stable and less reactive cyclic derivative (23). Substitutions in the β -position on the chain which would not be expected to interfere with the formation of intermediates (A-4, A-6) do not alter activity, while those which would prevent intermediate formation cause inactivation (A-7, A-8).

The inactivity of compounds B-2 and B-3 can be readily explained on the basis that a side-chain containing a tertiary amine may act to prevent the formation of active intermediates. It is known that alkyl halides attached to tertiary amines are capable of forming stable, 6-membered heterocyclic derivatives (24). In both B-2 and B-3 the nitrogen of the substituent is in such a position as to form a 6-membered ring by cyclization with the β -chloroethyl grouping. Such a reac-

tion would prevent formation of the intermediate required for activity. Although a representative compound has not been tested, it may be anticipated on the basis of the reduced tendency for the analogous formation of larger rings (24) that a γ -propylamine substitution would have a less deleterious effect on adrenergic blocking activity.

The third requirement is the most complex, but data bearing on this point (table 3) offer the deepest insight into the marked chemical and pharmacological specificity of members of the Dibenamine series of blocking agents. The fact that all compounds with saturated substitutions are inactive is obviously in accord with the concept that activity is dependent upon stabilizing resonance. Substitutions on the aromatic ring itself which interfere with, but probably would not completely prevent stabilization through hyperconjugation, cause relative inactivation. It is of particular interest to note that chlorine substituted in the 4-position (C-30) results in markedly reduced activity while the same substitution in the 3-position (C-32), where it would not interfere with and might even favor conjugation, actually enhances activity. The same relationship appears to hold for methyl groups, but the effect is less pronounced, probably because of the much smaller inductive effect of the methyl substituents. Methyl substitutions in the 4- (C-19) and especially in the 2-position (C-21) cause a delayed onset of action, while the 3-substitution (C-20) does not significantly alter activity. It is possible that steric effects are an added factor in the reduced activity of 2-substituted compounds, but the difference between the 3- and 4-substituted compounds finds ready explanation only on the basis of altered resonance.

A consideration of the effect of altering the number of atoms between the aromatic ring and the nitrogen also lends support to this interpretation. The direct apposition of the ring and the nitrogen, which would reduce but not prevent resonance stabilization, yields a compound which has the smallest detectable activity (C-8). The interposition of one carbon atom permits effective resonance and all such compounds are effective blocking agents if they fulfill the other requirements for activity. Further additions between the aromatic ring and the nitrogen also agree with the theory. The presence of two carbons, which would inhibit resonance, markedly reduces activity (C-9). The activity of C-10 is somewhat greater than might be expected in view of the considerable insulating effect of the two-carbon chain. However, the much reduced stabilizing effect of the β -phenylethyl grouping as compared to the benzyl is reflected by the fact that substitution of a benzyl for the short alkyl moiety of compounds of the β -phenylethyl type leads to a marked increase in activity (25).

Addition of an oxygen to produce phenoxyethyl derivatives, which favors hyperconjugation, produces highly active compounds (C-14 to C-17). The γ -phenylpropyl derivatives (C-11, C-12) are probably inactive because of the magnitude of the insulating effect of the saturated 3-carbon chain, an effect which would be considerably greater than that of the oxyethyl grouping. This interpretation is strengthened by the activity of the cinnamyl analog (C-13) of compound C-12 in which the β - γ unsaturation of the propylene chain would markedly reduce the energy requirements for hyperconjugation.

Although fundamentally different in structure from other members of this

series, compound D-1 satisfactorily fulfills the requirements for activity listed above, including the presence of an aromatic nucleus in a position to stabilize the intermediate in the same way as do benzyl and other active groupings attached to the nitrogen.

The fourth requirement is necessitated by the complete inactivity of all benzyl derivatives having a chain of two or more carbon atoms attached to the aromatic ring. These substitutions have a much smaller inductive effect than chlorine, but result in complete rather than only partial inactivation (compare C-30 with C-34 and C-36, table 3). Such a sharp break between the methyl and ethyl substitutions would also not be expected on the basis of their effect on resonance. The fact that ethyl substitutions produce complete inactivity while the methoxy and piperonyl substitutions only reduce activity indicates that the plane of the substitution is important. The ethyl group would undoubtedly be held out of the plane of the ring through steric hindrance with the adjacent hydrogen atom, whereas the methoxy group would tend to be held in a coplanar configuration by the partial double bond character of the oxygen-ring carbon bond.

The marked difference in activity between the members of certain pairs of compounds (C-29 and C-30, C-31 and C-32, C-40 and C-41) is not completely explained. The extreme aqueous insolubility of compounds C-29, C-31 and C-40 may be important in their inactivity, particularly in the case of C-29 where even the soluble congener (C-30) is very weak. Whether a very low aqueous solubility provides a complete explanation for the lack of activity of C-31 and C-40 cannot be stated at this time.

From the above discussion it is apparent that all or almost all the structure-activity data obtained on this series of compounds are explainable on the assumption that the β -haloalkyl group is primarily involved in the adrenergic blocking activity, and that it produces its blocking effects through certain intermediates formed in the body. The marked influence of relatively minor substitutions elsewhere in the molecule appears to be best explained by their effect on the hyperconjugation by which the aromatic or unsaturated heterocyclic constituents of the molecule stabilize the reactive intermediates. In a few cases an additional steric factor appears to preclude activity although all other requirements have been met. In the presence of a β -haloalkyl and one benzyl group, the third substituent on the nitrogen is relatively unimportant as long as a tertiary amine is formed and the substituent itself does not undergo a reaction which interferes with reactions of the β -haloalkyl moiety. In the presence of certain other unsaturated ring structures (e.g. fluorenyl, C-43) the nature of the third substituent has more effect on activity (8).

No generalizations which cover the entire series can be made regarding the relation of structure to toxicity, but certain intermediate compounds give an indication of the basis for the much reduced toxicity of Dibenamine and related active compounds in comparison with nitrogen mustards such as C-3 and C-4. The first factor is the presence of only a single β -chloroethyl group. The effect of this factor upon toxicity is clearly illustrated by the marked reduction in the acute toxicity of C-2 as compared to that of C-3. It also explains the fact that

Dibenamine does not damage hemopoietic tissue (2). A second factor of importance is the low aqueous solubility of these compounds. This may be noted in the parallel decrease in toxicities and solubilities found in compounds with progressively longer aliphatic substituents (B-1 to B-10). The effect of decreased aqueous solubility is limited, however, as can be seen by comparing compounds C-3 and C-4. In addition to the above factors, benzyl and other substitutions producing adrenergic blocking activity have a marked specific action in reducing toxicity. For example compound B-10 is considerably less soluble than Dibenamine, but it is more than twice as toxic, and compound B-11 is very much less toxic than C-4 although their aqueous solubilities are not very different. The mere presence of an aromatic ring is not adequate to produce this effect. Compound C-9 is much more toxic than Dibenamine although it possesses two β -phenylethyl groups and also has a lower aqueous solubility. This specific action of benzyl and other properly placed unsaturated substituents is probably related to the resonance stabilization which such groupings provide. The ethylenimmonium derivatives are largely responsible for the toxicities of the nitrogen mustards. Except for acute central nervous system stimulation observed after rapid intravenous injection (26), it appears that most of the toxicity of members of the Dibenamine series is dependent upon similar ethylenimmonium intermediates. Any reduction in the reactivity of such intermediates through resonance stabilization might therefore be expected to reduce toxicity.

It should be specifically pointed out that the above structural requirements for activity apply *only to members of the β -haloalkylamine series producing a Dibenamine-type blockade*, i.e. a prolonged, non-equilibrium blockade produced through intermediates which react with thiosulfate. There are several types of adrenergic blocking agents to which the above principles obviously do not apply such as the ergot alkaloids, yohimbine, imidazolines, tetrahydro-isoquinolines, (D-7), etc. However, in certain other series of blocking agents it is not so obvious that a different mechanism is involved. Recent reports by Kahane and Lévy (27) have renewed interest in phenoxyethyl primary and secondary amines which produce adrenergic blockade. These are closely related to compounds C-14 to C-17 of the present series and their activity would appear to contradict the above statements regarding the necessity of both the tertiary amine structure and the β -haloalkyl substituent. However, we have found that these compounds exert a very different type of blocking action from that characteristic of Dibenamine. The blockade is transitory and is not significantly altered by the presence of thiosulfate. It has also been demonstrated that C-16 possesses both types of blocking activity for in the presence of thiosulfate the blockade produced by this agent is very similar to that characteristic of its hydrolysis product (the β -hydroxyethylamine) and of the corresponding secondary amine. The phenoxyethyl moiety apparently has special steric significance in the production of adrenergic blockade. A detailed study of compounds containing this substituent will be published separately.

Although the structural requirements for adrenergic blocking activity in the β -haloalkylamine series discussed in this report cannot be considered as defini-

tive, they have been most helpful in directing the synthesis of new compounds. They also serve to focus attention upon the importance of the *chemical* properties of pharmacologically active agents, which in the past have been almost completely neglected in favor of *physical* (steric) factors.

Observations on the use of nitrogen mustards in the therapy of certain neoplasms (28-30) emphasize the need for a greater specificity of action. However, current attempts to improve the specificity of the anti-neoplastic action of certain β -haloalkylamines, *e.g.*, by attaching them to carcinogens (31) have been carried out with only a very limited knowledge of the chemical basis for the desired action and have met with little success. The fact that the highly specific adrenergic blocking action of the Dibenamine series of compounds can be accurately predicted on the basis of almost equally specific requirements of chemical structure suggests that a careful study may reveal relationships which will allow for the preparation and selection of β -haloalkylamines with equally specific but different pharmacological properties.

SUMMARY

Certain chemical and pharmacological properties of 113 compounds chemically related to Dibenamine are presented and discussed as representative of a much larger series studied in the same manner.

It is concluded that the highly specific adrenergic blocking activity of members of the Dibenamine series can be adequately explained on the basis of a few specific requirements of chemical structure. To be active a compound must meet the following requirements:

1. It must be a tertiary amine or the quaternary derivative of an active amine.
2. It must include at least one β -haloalkyl group capable of forming an intermediate ethylenimmonium (or vinyl) derivative.
3. It must include an unsaturated ring substituent attached to the amine in such a way as to allow resonance stabilization of the active intermediate.
4. In the case of benzyl derivatives, there must be no substitution on the phenyl ring which tends to be out of the plane of the ring.

Compounds meeting these conditions were found to be active, and no compound failing to meet all these requirements could be shown to produce significant Dibenamine-type adrenergic blockade.

The very low toxicity of Dibenamine and most of its active congeners appears to be due to three factors:

1. The presence of only one β -haloalkyl moiety.
2. Their low aqueous solubility.
3. The reduced reactivity of their ethylenimmonium (or vinyl) intermediates as a result of resonance stabilization.

Although the chemical basis for activity outlined in this report cannot be considered as definitive, it has proved most helpful in directing the synthesis of a large number of active compounds and should provide useful direction in the development of new β -haloalkylamine adrenergic blocking agents of experimental and clinical value. It should also serve to focus attention upon the im-

portance of the *chemical* properties of pharmacologically active agents, which in the past have been almost completely neglected in favor of *physical* (steric) properties.

REFERENCES

1. NICKERSON, M., AND GOODMAN, L. S.: *Proc. Am. Fed. Clin. Res.*, **2**: 109, 1945.
2. NICKERSON, M., AND GOODMAN, L. S.: *THIS JOURNAL*, **89**: 167, 1947.
3. NICKERSON, M., AND NOMAGUCHI, G. M.: *THIS JOURNAL*, **93**: 40, 1948.
4. NICKERSON, M., AND GOODMAN, L. S.: *Fed. Proc.*, **7**: 397, 1948.
5. NICKERSON, M., NOMAGUCHI, G., AND GOODMAN, L. S.: *Fed. Proc.*, **6**: 195, 1946.
6. LOEW, E. R., AND MICETICH, A.: *THIS JOURNAL*, **93**: 434, 1948.
7. HUNT, C. C.: *THIS JOURNAL*, **95**: 177, 1949.
8. KERWIN, J. F., AND ULLYOT, G. E.: Abstracts. 115th Meeting, Am. Chem. Soc., p. 10K, 1949.
9. PFEIFFER, C. C.: *Science*, **107**: 94, 1948.
10. CLARK, A. J.: *J. Physiol.*, **61**: 547, 1926.
11. MENDEZ, R.: *THIS JOURNAL*, **32**: 451, 1928.
12. WELLS, J. A., MORRIS, H. C., BULL, H. B., AND DRAGSTEDT, C. A.: *THIS JOURNAL*, **85**: 122, 1945.
13. ACHESON, G. H., AND FARAH, A.: Personal communication.
14. NICKERSON, M., AND NOMAGUCHI, G. M.: *THIS JOURNAL*, **95**: 1, 1949.
15. GOLUMBIC, C., FRUTON, J. S., AND BERGMANN, M.: *J. Org. Chem.*, **11**: 518, 1946.
16. FRUTON, J. S., AND BERGMANN, M.: *J. Org. Chem.*, **11**: 543, 1946.
17. STAHMANN, M. A., FRUTON, J. S., AND BERGMANN, M.: *J. Org. Chem.*, **11**: 704, 1946.
18. STAHMANN, M. A., GOLUMBIC, C., STEIN, W. H., AND FRUTON, J. S.: *J. Org. Chem.*, **11**: 719, 1946.
19. STAHMANN, M. A., AND BERGMANN, M.: *J. Org. Chem.*, **11**: 586, 1946.
20. FRUTON, J. S., STEIN, W. H., AND BERGMANN, M.: *J. Org. Chem.*, **11**: 559, 1946.
21. FRUTON, J. S., STEIN, W. H., STAHMANN, M. A., AND GOLUMBIC, C.: *J. Org. Chem.*, **11**: 571, 1946.
22. GOLUMBIC, C., FRUTON, J. S., AND BERGMANN, M.: *J. Org. Chem.*, **11**: 518, 1946.
23. GIBBS, C. F., AND MARVEL, C. S.: *J. Am. Chem. Soc.*, **56**: 725, 1934.
24. LITTMANN, E. R., AND MARVEL, C. S.: *J. Am. Chem. Soc.*, **52**: 287, 1930.
25. ULLYOT, G. E., KERWIN, J. F., AND FELLOWS, E. J.: Personal communication.
26. NICKERSON, M.: *Endocrinology*, **44**: 287, 1949.
27. KAHANE, E., AND LÉVY, J.: *Bull. Soc. Chem. Biol.*, **27**: 562, 1945.
28. GOODMAN, L. S., WINTROBE, M. M., DAMESHEK, W., GOODMAN, M. J., GILMAN, A., AND McLENNAN, M. T.: *J. A. M. A.*, **132**: 126, 1946.
29. JACOBSON, L. O., SPURR, C. L., BARRON, E. S. G., SMITH, T., LUSHBAUGH, C., AND DICK, G. F.: *J. A. M. A.*, **132**: 263, 1946.
30. RHOADS, C. P.: *J. A. M. A.*, **131**: 656, 1946.
31. FRIEDMAN, O. M., AND SELIGMAN, A. M.: *J. Am. Chem. Soc.*, **70**: 3082, 1948.

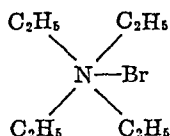
THE GANGLIONIC BLOCKING ACTION OF THIOPHANIAM DERIVATIVES

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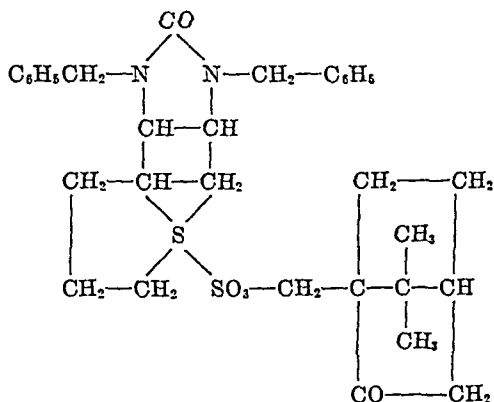
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The pharmacological action of some thiophanium derivatives has been found similar in many respects to that of tetraethylammonium bromide (TEA). Acheson and Moe (1) ascribed the vasodepressor action of tetraethylammonium bromide to a blocking effect on sympathetic ganglia. The ganglionic blocking action was demonstrated on the superior cervical, the stellate and the vagus ganglia. It has now been found that certain sulfonium salts have much the same qualitative action as the quaternary ammonium salt and one of the thiophanium compounds (Nu-2222) has a much more powerful and longer action than TEA as a vasodepressor and ganglionic blocking agent. The formula for TEA and Nu-2222 are as follows:



TEA

Tetraethyl Ammonium
Bromide



Nu-2222

d-3,4(1',3'-dibenzyl-2'-keto-imidazolido)-1,2-
trimethylene thiophanium d-camphor sulfonate

The thiophanium derivatives were synthesized in the Scientific Department of these laboratories by L. H. Sternbach, M. W. Goldberg and S. Kaiser. Nu-2222 is available from the Roche synthesis of biotin.

Circulation: The intravenous vasodepressor effects of the compounds were measured on cats and dogs anesthetized with Dial-urethane and on two monkeys anesthetized with pentobarbital sodium. Arterial pressure was recorded with a mercury manometer. The average results in cats are summarized in table 1. In 27 cats, Nu-2222 had an average of 33 times the potency of TEA and 3 times the duration of depressor effects. In seven dogs, Nu-2222 had approximately 30 times the potency and twice the duration of TEA as a depressor agent. In two

¹ Deceased.

monkeys, Nu-2222 had approximately 30 times the potency of TEA and slightly longer duration.

By the intramuscular route in two cats and four dogs, doses of Nu-2222 up to 0.33 mgm./kgm. were necessary to produce depressor effects. Thus about 20 times the effective intravenous dose was required to produce vasodepression by the intramuscular route.

By the oral route in three cats and six dogs, the effects of Nu-2222 were erratic, doses of the order of 8 mgm./kgm. or more being necessary to produce depressor effects. This thiophanium derivative is, therefore, not readily absorbed.

TABLE I
Vasodepressor and Ganglionic Blocking Action of TEA and Thiophanium Salts in Cats

COMPOUND	DOSE	ARTERIAL PRESSURE		NICITATING MEMBRANE	
		Decrease	Duration	% Inhibition	Duration
	mgm./kgm	mm Hg	minutes		minutes
TEA	.25	22	2	37	2
	.5	27	3	50	3
	1	43	4	56	4
	2	45	8	77	8
	4	51	9	90	9
Nu-2222	.015	32	8	41	8
	.03	42	11	78	11
	.06	51	20	83	21
	.12	66	23	83	35
	.25	78	32	83	38
Nu-2221	.03	18	5	46	8
	.06	21	7	85	7
	.12	28	7	75	25
	.25	50	9		
	.12	16	2	45	7
Nu-1994	.25	32	4	78	11
	.50	44	4	91	25
Nu-2356	1	36	2	13	2
	2	48	4	15	2
	4	20	4	20	4

The l-isomer, Nu-2221, was approximately one-half as potent a depressor agent as the d-isomer, Nu-2222. The monobenzyl derivative, Nu-1994, 3,4-(monobenzyl-2'-keto-imidazolido)-1,2-trimethylene-thiophanium chloride, was about twice as active as TEA and thus much less active than the dibenzyl derivative. The unsubstituted derivative, Nu-2356, 3,4-(2'-keto-imidazolido)-1,2-trimethylene-thiophanium bromide, was only slightly active.

Ganglionic Transmission: The effects on transmission through the superior cervical ganglion of cats were measured by the method described by Acheson and Moe (1). The superior cervical nerve of cats, anesthetized with Dial-urethane, was stimulated through shielded electrodes with an Electrodyne stimulator.

Maximal shocks were given at the rate of 3-5 per second to obtain a sustained contraction of the nictitating membrane. The membrane was attached to a lever via a thread. The per cent decrease in height of contraction after intravenous administration of a compound was calculated. The results are summarized in table 1. A typical record comparing the effect of Nu-2222 and TEA on the nictitating membrane and arterial pressure responses in a cat is shown in figure 1.

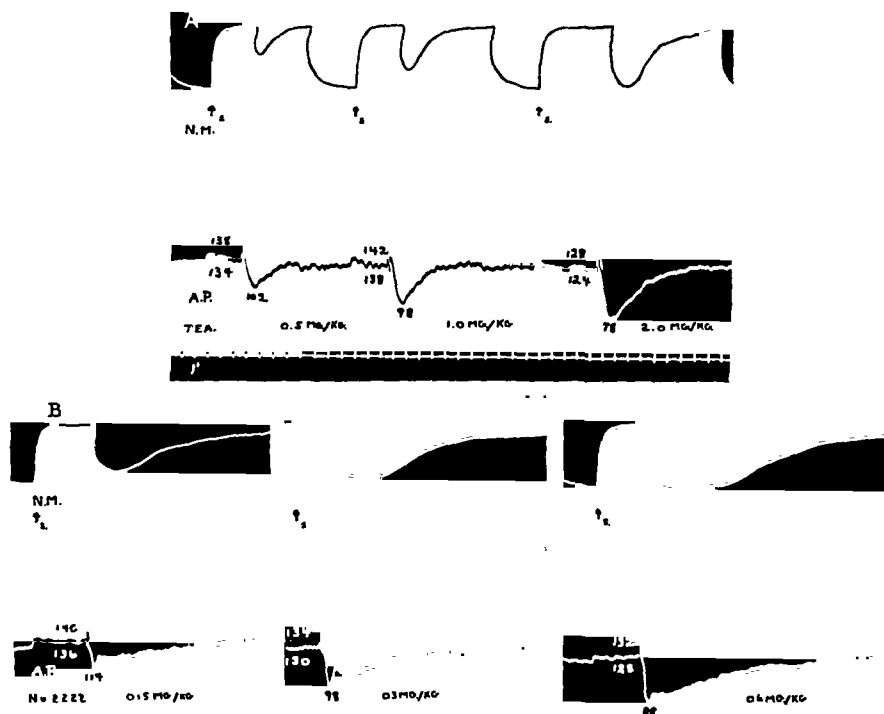


FIG. 1. COMPARISON OF THE EFFECT OF TEA AND NU-2222 ON THE CONTRACTIONS OF THE NICTATING MEMBRANE PRODUCED BY PREGANGLIONIC NERVE STIMULATION AND ON ARTERIAL PRESSURE

Cat, Dial-urethane anesthesia.

A—Nictitating membrane; S—preganglionic stimulation of the cervical sympathetic nerve with maximal shocks at the rate of 5 per second. Arterial pressure. Numbers refer to mm. Hg. Time in minutes. TEA 0.5 mgm./kgm., 1.0 mgm./kgm. and 2.0 mgm./kgm. given intravenously.

B—N.M. = Nictitating membrane. A. P. = Arterial pressure. Nu-2222 .015 mgm./kgm., .03 mgm./kgm. and .05 mgm./kgm. given intravenously.

The potency and duration of effects of the compounds on the nictitating membrane paralleled very closely the arterial pressure effects. Thus Nu-2222 was approximately 30 times as strong as TEA, Nu-2221 about 15 times as strong, Nu-1994 about twice and Nu-2356 was relatively inactive. The duration of effects on the nictitating membrane also paralleled closely the depressor effects.

A demonstration that the effects of Nu-2222 and TEA are exerted at the ganglion of the sympathetic pathway and not on the nictitating membrane is shown

in figure 2. When the superior cervical ganglion was crushed and the postganglionic fibers stimulated, TEA and Nu-2222 had no effect on the membrane responses although the usual depressor effects on arterial pressure were observed. Nu-2222, therefore, resembles TEA in having primarily a ganglionic blocking action.

The inhibitory action of TEA and Nu-2222 on the cardiodepressor response to the stimulation of the peripheral end of the vagus nerve and to the vasopressor response to carotid occlusion in the dog is illustrated in figure 3. As Reardon

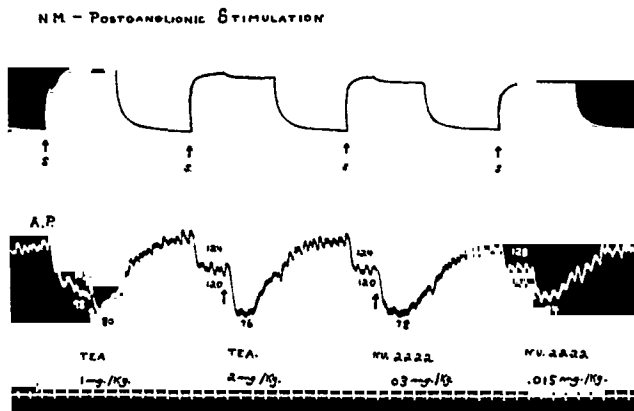


FIG. 2. COMPARISON OF THE EFFECT OF TEA AND Nu-2222 ON THE CONTRACTIONS OF THE NICTITATING MEMBRANE PRODUCED BY POSTGANGLIONIC STIMULATION AND ON ARTERIAL PRESSURE

Cat, Dial-urethane anesthesia. S—Postganglionic stimulation of the cervical sympathetic nerve with ganglion crushed. A.P. = Arterial pressure. Numbers refer to mm. Hg. Time in minutes.

From left to right, TEA, 1 mgm./kgm. TEA, 2 mgm./kgm. Nu-2222, 0.03 mgm./kgm., Nu-2222, 0.015 mgm./kgm. Arrows indicate time of injections.

et al. (2) found with TEA, Nu-2222 blocks transmission through the vagus ganglion and also blocks the reflex pressor effect produced by carotid occlusion. These blocking effects disappear gradually as the arterial pressure returns to normal. Similar blocking effects were observed in cats and monkeys.

Action of Neostigmine at Ganglia: Reardon *et al.* (2) observed that neostigmine (Prostigmin) abolished the hypotensive effects of TEA in dogs and counteracted the blocking action of TEA on the carotid sinus reflex and on the vagus inhibition of the heart. Neostigmine in doses of 40, 80, 160 and 320 microgm./kgm., intravenously, caused a progressive inhibition of the arterial pressure changes produced by moderate doses of TEA and Nu-2222.

Figure 4 illustrates that a block of sympathetic ganglia produced by a large dose of Nu-2222 is effectively counteracted by neostigmine. The profound fall in arterial pressure and the complete block of transmission through the superior cervical ganglion was overcome by a large dose of neostigmine. This evidence indicates that neostigmine can stimulate ganglia at the locus of the block by TEA or Nu-2222.

Ephedrine as an Antidote for TEA and Nu-2222: The typical depressor and ganglionic blocking effects of TEA and Nu-2222 are practically abolished by ephedrine, 1 mgm./kgm. intravenously. The sustained contraction of the nic-

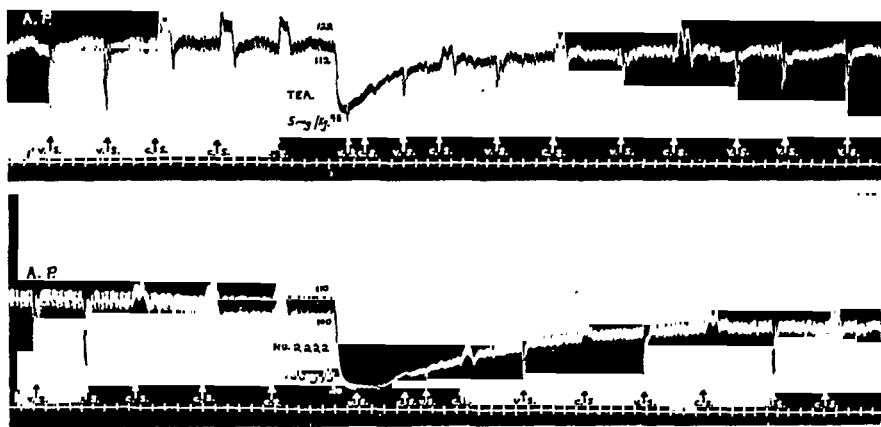


FIG. 3. EFFECT OF TEA AND NU-2222 ON THE CARDIODEPRESSOR RESPONSE TO VAGUS STIMULATION AND ON THE VASOPRESSOR RESPONSE TO THE OCCLUSION OF THE CAROTID ARTERY

Dog, Dial-urethane anesthesia. A. P. = Arterial pressure. Numbers refer to mm. Hg. V. S. = Maximal stimulation of peripheral end of vagus for 10 seconds. C. S. = Clamping of carotid artery for one minute. Time in minutes.

Upper record—TEA, 5 mgm./kgm.

Lower record—Nu-2222, 0.06 mgm./kgm., intravenously.

titating membrane produced by ephedrine is not relaxed by TEA and these substances are ineffective on the arterial pressure during the pressor response to ephedrine. An illustration of the antidotal action of ephedrine is given in figure 5. The markedly low arterial pressure produced by a relatively large dose of Nu-2222 in a dog is returned sharply to the normal level by 1 mgm./kgm. of ephedrine. The inhibition of the effects of vagus stimulation on arterial pressure also disappears when the arterial pressure becomes normal. These experiments demonstrate that ephedrine is an effective antidote for the hypotensive action of TEA and Nu-2222.

Curare-like Action of TEA and Nu-2222: The action of TEA and Nu-2222 on neuromuscular transmission was measured in three dogs in comparison with tubocurarine Cl. The method described by Lehmann (3) was used for measuring cu-

Intestinal motility was measured in dogs anesthetized with Dial-urethane and the record of jejunal activity was obtained with a balloon connected to a tambour. TEA, 2 mgm./kgm., and Nu-2222, 0.06 mgm./kgm., had only slight effects on jejunal motility. The slight decrease in intestinal tone was accompanied by a moderate fall in arterial pressure.

Nu-2222 was inactive as a spasmolytic on isolated intestine. Concentrations of 10^{-4} gm./cc. did not relax the spasms produced by acetylcholine, histamine or barium chloride.

TABLE 2
Toxicity of TEA and the Thiophanium Salts

COMPOUND NO.	SPECIES	ROUTE	LD ₅₀ ± S.E.
			mgm./kgm.
TEA	Mouse	i.v.	38 ± 3.4
	Mouse	i.p.	60 ± 8
	Mouse	p.o.	> 2000
	Rat	i.v.	63 ± 5
	Rat	i.p.	115 ± 34
	Rabbit	i.v.	72 ± 8
	Dog	i.v.	55
2222	Mouse	i.v.	21 ± .1
	Mouse	i.p.	140 ± 10
	Mouse	p.o.	1250 ± 87
	Rat	i.v.	21 ± 3
	Rat	i.p.	270 ± 57
	Rabbit	i.v.	23 ± 2
	Dog	i.v.	0.75
	Dog	i.m.	8
	Dog	p.o.	400
	Guinea pig	i.v.	13 ± 2
2221	Monkey	i.v.	> 8
	Mouse	i.v.	23 ± 3
	Mouse	i.p.	135 ± 9
1994	Mouse	i.p.	263 ± 26
2356	Mouse	i.p.	594 ± 120

Toxicity: The acute toxicity of the thiophanium compounds in comparison with TEA varies greatly not only among various routes but also among species. The results are summarized in table 2. By the intraperitoneal route in mice, Nu-2222 and Nu-2221 are half as toxic as TEA, Nu-1994 is 1/4 as toxic and Nu-2356 is 1/10 as toxic. When the camphor sulphonate ion in Nu-2222 is replaced by the chloride ion, the toxicity is not changed. By the intravenous route in mice, Nu-2222 and Nu-2221 are twice as toxic as TEA and Nu-2222 is also more toxic in rats and rabbits. By the oral route, Nu-2222 is more toxic than TEA in mice. Nu-2222 has an unusually high toxicity in dogs. Nu-2222 and TEA were not toxic to two monkeys at doses up to 8 mgm./kgm. I.V. TEA produced death by respiratory failure in all species and Nu-2222 produced a similar death in all species except the dog. In the latter it produced death by a hemorrhagic disorder.

Hemorrhages in the gastro-intestinal tract and petechiae in internal organs were observed at autopsy. Such abnormalities were not found in any other species.

The clotting times were measured on blood withdrawn from the jugular vein and placed in a dry test tube. It was found that doses of Nu-2222 of 0.25 to 1.0 mgm./kgm. I.V. prolonged clotting time. Samples of blood withdrawn after 1/2, 1 and 2 hours did not clot in 8 hours. Doses of 0.1 mgm./kgm. I.V. did not prolong clotting time. Also doses of 2.5 to 10 mgm. intramuscularly and doses of 50 to 200 mgm./kgm. orally did not prolong clotting times. Doses of TEA of 25 mgm./kgm. intravenously did not change the clotting time of dogs. In rabbits, doses of Nu-2222 of 10 mgm./kgm. intravenously did not prolong clotting times. In two monkeys a dose of Nu-2222 of 8 mgm./kgm. intravenously and of TEA of 8 mgm./kgm. did not prolong clotting time nor produce hemorrhagic effects. There were no significant changes in the red and white cell counts, hemoglobin or platelet counts.

It is probable that the hemorrhagic disorder and the prolonged clotting time in dogs, accompanying the intravenous administration of toxic doses of Nu-2222, are produced by the profound fall in blood pressure which results in a state of shock. The lethal effects can be modified by phenindamine (Thephorin) (8) an antihistamine agent and by toluidine blue, an antiheparin agent. Phenindamine, at a dose of 1 mgm./kgm. intravenously, protected three of four dogs given 4 mgm./kgm. I.V. of Nu-2222 and a dose of 2.5 mgm./kgm. protected three of four dogs given 10 mgm./kgm. I.V. of Nu-2222. However, the clotting times were prolonged and some hemorrhagic symptoms were still observed. Toluidine blue, at a dose of 10 mgm./kgm. intravenously protected two of three dogs given 4 mgm./kgm. I.V. of Nu-2222 and prevented the change in clotting time but not the hemorrhagic effects. Thephorin, 2.5 mgm./kgm. and toluidine blue, 10 mgm./kgm., given together, protected two of three dogs when a dose of 10 mgm./kgm. of Nu-2222 was given. The clotting time changes and the hemorrhagic effects were also prevented. These results indicate that Nu-2222 releases histamine and heparin in the state of shock and the lethal and hemorrhagic effects of these substances can be modified by antihistamine and antiheparin agents.

DISCUSSION. It has been found that certain thiophanium salts have the same qualitative ganglionic blocking effects as the quaternary ammonium salt, tetraethylammonium bromide. The d-form of the thiophanium derivative, Nu-2222, has a vasodepressor potency about 30 times that of TEA and several times the duration in dogs, cats and monkeys. Similar to the effects of TEA, Nu-2222 blocks both sympathetic and parasympathetic ganglia. Vagus action and carotid sinus reflexes are inhibited. Gastric and intestinal motility are inhibited *in vivo* but the isolated intestine is not affected. The vasodepressor and ganglionic blocking action of TEA and Nu-2222 are counteracted by large doses of neostigmine. This action of neostigmine is probably due to a nicotine-like stimulatory action of the quaternary ammonium group. The thiophanium salt may be displaced from its locus of action by the more powerful quaternary ammonium ion. On the other hand, the action of ephedrine in overcoming the vasodepressor action

of TEA and Nu-2222 is probably a peripheral effect. Nu-2222 like TEA does not have a curare-like blocking action on neuromuscular transmission. Although straited muscle action is not inhibited, the compounds produce their lethal effects by respiratory depression.

In most species the toxicity of Nu-2222 by the intravenous route is two to four times that of TEA but since it has an activity 30 times that of TEA, the safety margin is high. In the dog Nu-2222 is about 75 times as toxic as TEA so that the safety factor is lower than that of TEA. In the dog, Nu-2222 produces a hemorrhagic disorder characterized by a prolonged clotting time and hemorrhages into internal organs. This effect is probably due to the release of histamine and heparin since the lethal effects are modified by antihistamine and antiheparin agents. The dog is also unusually sensitive to acetyl- β -methyl-choline. This material in low doses produces a bloody diarrhea in dogs (8). Peptone shock in dogs is accompanied by the release of histamine and the effects can be counteracted by antihistamine agents (9). In the dog x-irradiation also liberates heparin and histamine and the hemorrhagic and lethal effects can be modified with antiheparin and antihistamine agents (10, 11). The mechanism of release of these tissues elements is still unknown. Although we found no hemorrhagic tendencies with TEA, it should be noted that Ham (13) observed purpura in a human subject following treatment with TEA.

SUMMARY

Nu-2222 is a thiophanium derivative which has the characteristic ganglionic blocking and hypotensive effects of tetraethylammonium bromide. It has about 30 times the potency and twice the duration of TEA in the dog, cat and monkey. It blocks transmission through the superior cervical ganglion, vagus action on the heart and the carotid sinus pressor reflex. The hypotensive effects are counteracted by ephedrine and large doses of neostigmine. It has no curariform action. It has only a slight effect on intestinal tone but inhibits gastric motility in large doses. It is two to four times as toxic as TEA intravenously in mice, rats and rabbits but 75 times as toxic in dogs. The lethal and hemorrhagic effects in dogs can be modified to a large extent by antihistamine and antiheparin agents.

REFERENCES

1. ACHESON, G. H., AND MOE, G. K.: *THIS JOURNAL*, 87: 220, 1946.
2. REARDON, M. J., MARZONI, F. A., AND HENDRIX, J. P.: *Fed. Proc.*, 6: 364, 1947.
3. LEHMANN, G.: *Barell Jubilee Volume*, 314, 1946.
4. ACHESON, G. H., AND PEREIRA, S. A.: *THIS JOURNAL*, 87: 273, 1946.
5. DODDS, D. C., OULD, C. L., AND DAILEY, M. E.: *Gastroenterology*, 10: 1007, 1948.
6. BROWN, H. S., POSEY, E. L., JR., AND GAMBILL, E. E.: *Gastroenterology*, 10: 837, 1948.
7. ZWEIG, M., STEIGMANN, F., AND MEYER, K. A.: *Gastroenterology*, 11: 200, 1948.
8. LEHMANN, G.: *THIS JOURNAL*, 92: 249, 1948.
9. WENER, J., HOFF, H. E., AND SIMON, M. A.: *Gastroenterology*, 11: 904, 1948.
10. DAVIS, J. C., JR., AND HATERIUS, H. O.: *Proc. Soc. Exper. Biol. and Med.*, 70: 275, 1949.
11. ELLINGER, F.: *Science*, 104: 502, 1946.
12. ALLEN, J. G., AND JACOBSON, L. O.: *Science*, 105: 388, 1947.
13. HAM, F. F.: *California Med.*, 69: 279, 1948.

THE FATE OF ACETOPHENETIDIN (PHENACETIN) IN MAN AND METHODS FOR THE ESTIMATION OF ACETOPHENETIDIN AND ITS METABOLITES IN BIOLOGICAL MATERIAL^{1, 2}

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Recent work in this laboratory has shown that acetanilide is metabolized in man through two routes (1). The major one involves oxidation to N-acetyl p-aminophenol. It was suggested that the analgesic effect of the parent drug is directed mainly through this compound. A minor route of metabolism involves deacetylation to yield aniline. This compound was shown to be the precursor of the substance, probably phenylhydroxylamine, which is responsible for the formation of methemoglobin. The similarity in structure of acetophenetidin (p-ethoxy acetanilide) and acetanilide suggested that acetophenetidin might be metabolized through similar routes.

Work previously reported on the fate of acetophenetidin in the body is meager and suggests that the compound is first deacetylated to p-phenetidin (p-ethoxy aniline) which is then deethylated to p-aminophenol, the latter compound being then excreted combined with sulphuric or glucuronic acid (2).

The investigation described here concerns the physiological disposition and chemical fate of acetophenetidin in man and the pharmacological activity of its transformation products.

CHEMICAL METHODS. Sensitive methods for the estimation of acetophenetidin and its metabolic products were required in the study of the physiological disposition of acetophenetidin. These methods are described below.

Determination of acetophenetidin: In the method reported below, acetophenetidin is isolated from biological material by extraction into benzene. The benzene is evaporated and the residue taken up in acid. The acetophenetidin is hydrolyzed and the resulting p-phenetidin diazotized and coupled with alpha naphthol. The dye formed is extracted into a small volume of benzene, the benzene solution acidified with trichloroacetic acid and the dye assayed at 600 mu in a spectrophotometer.

Procedure: Add 1 to 5 cc. of biological material³ at pH 6 or higher (sample containing up to 10 microgm. of acetophenetidin) to a 60 cc. glass-stoppered bottle containing 30 cc. of isoamyl alcohol-benzene.⁴ Dilute the aqueous phase to 5 cc. if its volume is smaller than this.

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² Presented in part at the meeting of the Federation of American Societies for Experimental Biology held at Atlantic City, New Jersey, in March, 1948. (Fed. Proc., 7: 207, 1948).

³ Organ tissues and feces are prepared for analysis by emulsification in acid as described in a previous paper (3).

⁴ A technical grade of benzene is purified by successive washings with 1N NaOH, 1N HCl and three washings with water. Isoamyl alcohol (1.5 per cent by volume), reagent grade, which has been similarly treated is added to each solvent to minimize the adsorption of the compound from the solvent onto glass surfaces.

Shake for ten minutes and centrifuge the bottle. Transfer 20 cc. of the solvent phase to a 50 cc. wide-mouthed graduated conical tube and evaporate on a boiling water bath to approximately 0.25 cc. A stirring rod in the tube aids evaporation. Add approximately 7 cc. of 0.5 *N* HCl and heat on a boiling water bath for 45 minutes. Cool and adjust the volume with water to exactly 6 cc. Transfer 5 cc. of the solution to a 15 cc. glass-stoppered conical centrifuge tube, add 0.5 cc. of 0.2 per cent sodium nitrite solution and let stand in the refrigerator for twenty minutes. Then add 0.5 cc. of 1 per cent ammonium sulfamate. Wait two minutes and add two drops of a 10 per cent solution of resublimed alpha naphthol in alcohol, followed by 1 cc. of 4*N* NaOH. Wait ten minutes¹ and add 0.5 cc. of concentrated HCl and 0.5 cc. of the isoamyl alcohol-benzene. Shake for one minute, thus extracting the dye into the benzene and then centrifuge. Introduce a finely drawn glass tube to the bottom of the lower aqueous layer and gently aspirate all but the last traces of water. This brings the solvent phase into the narrow portion of the tube, thus facilitating its subsequent transfer. Draw up by means of a capillary pipet and a rubber bulb 0.3 cc. of the solvent phase and transfer to a microcolorimeter tube. Add 0.1 cc. of a 25 per cent solution of trichloroacetic acid in ethylene dichloride. Mix by tapping the tube and determine the optical density of the solution at 600 mμ in the Coleman model 6 spectrophotometer adapted to microspectrophotometry (4). A reagent blank run through the procedure should not give an optical density of more than 0.020 when benzene plus trichloroacetic acid is used for the zero setting.²

The distribution of acetophenetidin in a benzene-water system is such that with volumes of 30 and 5 cc., respectively, about 95 per cent of the acetophenetidin is in the organic phase. Standards are therefore prepared by handling known amounts of acetophenetidin in the same manner as the unknowns. The optical densities are proportional to concentration. An optical density of approximately 0.080 is obtained in the adapted Coleman model 6 spectrophotometer when 1 microgm. of acetophenetidin is run through the procedure described above. Standards are run with each set of determinations since there is a small daily variation in the optical densities.

Acetophenetidin added to biological material in amounts from 1 to 5 microgm. was recovered with satisfactory precision (96 ± 5 per cent).

Determination of p-phenetidin (p-ethoxy aniline): p-Phenetidin is isolated from biological material by extraction in benzene, and returned to dilute acid. It is then diazotized and coupled with alpha naphthol. The resulting dye is extracted into a small volume of benzene, acidified with trichloroacetic acid and assayed at 600 mμ in a spectrophotometer.

Procedure: Add 1 to 5 cc. of biological material³ at pH 6 or higher (sample containing up to 10 microgm. of p-phenetidin) to 30 cc. of isoamyl alcohol-benzene⁴ in a 60 cc. glass-stoppered bottle. Shake for ten minutes and then centrifuge the bottle. Transfer 20 cc. of the benzene phase to a 60 cc. glass-stoppered bottle containing 6 cc. of 0.5*N* HCl. Shake for five minutes and centrifuge. Remove the benzene by aspiration. Transfer 5 cc. of the aqueous phase to a 15 cc. glass stoppered centrifuge tube and estimate the p-phenetidin as described in the acetophenetidin procedure.⁵ A reagent blank run through the procedure should not read more than 0.010 when benzene plus trichloroacetic acid is used for the zero setting.

Standards are prepared by taking 5 cc. of standard solution⁷ in 0.5*N* HCl and adding nitrite, sulfamate, coupling reagent and alkali followed by extraction of the acidified dye into benzene as described in the acetophenetidin procedure. A blank of 5 cc. of 0.5*N* HCl and the other reagents is used for the zero setting. The optical densities are proportional to concentration. An optical density of approximately 0.100 is obtained in the Coleman model 6 spectrophotometer when 1 microgm. of p-phenetidin is run through the procedure.

¹ In the determination of larger amounts of acetophenetidin or p-phenetidin (10 microgm. or more) which may be found in urine, and occasionally in plasma in the case of acetophenetidin, the optical density of the dye may be determined directly in the aqueous alkaline solution at a wave length of 500 mμ.

² Acetophenetidin values are corrected for p-phenetidin which may be present.

³ The p-phenetidin standard is prepared by dissolving 120 mgm. p-phenetidin in 5 cc. of ethyl alcohol and diluting to 1 liter with 0.1*N* HCl.

p-Phenetidin added to plasma, urine and homogenized organ tissues was recovered with satisfactory precision (95 ± 3 per cent).

N-acetyl *p*-aminophenol and *p*-aminophenol (free and conjugated) were estimated by methods previously described (5). *Methemoglobin* was determined by a slight modification of the method of Evelyn and Malloy (6).

Evidence for the identity of the substances determined in biological tissues: It was important to examine the specificity of the chemical methods since conclusions concerning the fate of acetophenetidin depended upon a knowledge of the identity of the substances measured in the biological material. The blanks in normal biological material were negligible. A technique, previously described by us, permits the identification of a substance being measured (3). It involves a comparison of the distributions of the substance with those of the authentic substance in a two-phase system consisting of an organic solvent and water at various pH values. The distribution of p-phenetidin, *N*-acetyl *p*-aminophenol and *p*-aminophenol (obtained after hydrolysis of conjugated forms) between an organic solvent and water at various pH values was compared with those of the apparent compounds extracted from urine. Acetophenetidin being a relatively neutral substance with a negligible dissociation

TABLE 1

Distribution of acetophenetidin and apparent acetophenetidin between water and various benzene-petroleum ether mixtures

The apparent acetophenetidin was obtained by benzene extraction of the urine of a subject receiving the drug. After washing with dilute HCl to remove p-phenetidin, the benzene phase was evaporated to dryness and the residue dissolved in water. Aliquots of this solution and of an authentic acetophenetidin solution were adjusted to pH 7 and extracted with equal volumes of the various benzene-petroleum ether mixtures. The fraction of the compounds extracted with the various solvent mixtures is expressed as the ratio of the amount of compound in the organic phase to total compound.

PER CENT PETROLEUM ETHER IN BENZENE	AUTHENTIC ACETOPHENETIDIN	APPARENT ACETOPHENETIDIN FROM URINE
0	0.95	0.93
25	0.85	0.83
50	0.66	0.61
100	0.15	0.16

at various pH values was compared with the apparent acetophenetidin from biological fluid in a two-phase system consisting of benzene and petroleum ether in various proportions and water at a constant pH.

The results with each substance indicated that within experimental error, the distributions of the apparent compounds from urine and the authentic compounds were the same (table 1, 2). It was concluded that the substances measured in biological fluids were identical with the authentic compounds.

FATE OF ACETOPHENETIDIN IN MAN. *Absorption and Excretion of Acetophenetidin.* Information concerning absorption from the gastro-intestinal tract and renal excretion of acetophenetidin was obtained from a balance study on two human subjects. The subjects were given acetophenetidin orally, 2 gm. daily in four divided doses for five consecutive days. Urine and stools were collected over the last 72 hours of administration. Less than 0.1 per cent of the dose was found in the stools, indicating that the absorption from the gastro-intestinal tract was essentially complete. Previous experiments had shown that the drug was not

destroyed after incubation in stool suspensions for 24 hours at 37° C. The urinary excretion amounted to only about 0.2 per cent of the administered acetophenetidin indicating that the drug underwent extensive metabolic change in the body.

TABLE 2

Distribution of apparent p-phenetidin, N-acetyl p-aminophenol, p-aminophenol and the authentic substances between an organic solvent and water at various pH values

The apparent compounds were extracted from the urine of subjects receiving acetophenetidin and returned to an aqueous phase as described under the chemical method for each compound. Aliquots of the aqueous phases were adjusted to various pH values and extracted with a solvent. Aqueous solutions of the authentic compounds were similarly treated. The aliquots of p-phenetidin were extracted with two volumes of benzene and those of p-aminophenol with eight volumes of ether. The N-acetyl p-aminophenol aliquots were saturated with sodium chloride and shaken with five volumes of ether. The fraction of the compounds extracted at various pH values is expressed as the ratio of the amount of compound in the organic phase to total compound.

pH	p-PHENETIDIN		N-ACETYL p-AMINOPHENOL		p-AMINOPHENOL	
	Authentic	Apparent	Authentic	Apparent	Authentic	Apparent
3.0	0.05	0.06			0.01	0.01
4.0	0.47	0.48	0.88	0.88	0.07	0.05
5.0	0.90	0.89			0.37	0.37
6.0					0.70	0.70
7.0	0.95	0.93	0.88	0.93	0.74	0.74
9.0			0.89	0.89		
10.0			0.79	0.75		
11.0			0.62	0.58		
13.0			0.00	0.00		
(0.01N NaOH)						

TABLE 3

Plasma acetophenetidin and per cent methemoglobin following oral administration of 1.2 and 2 gram doses of acetophenetidin to man

TIME	SUBJECT A (1.2 GM.)		SUBJECT B (2.0 GM.)		SUBJECT C (2.0 GM.)	
	Aceto-phenetidin	Methemo-globin	Aceto-phenetidin	Methemo-globin	Aceto-phenetidin	Methemo-globin
hrs.	mgm./L.	per cent	mgm./L.	per cent	mgm./L.	per cent
1	0.7	0.3	4.0	1.2	1.4	0.6
2	2.2	0.8	4.0	1.2	0.8	0.6
3	0.7	1.3	2.2	2.4	0.3	3.3
5	0.2	1.0	1.1	1.9	0.05	1.4
8	0.1	1.3	0.2	1.7	0.0	1.4

Plasma concentration—time curves. Plasma concentrations of acetophenetidin and per cent methemoglobin were measured after the administration to man of single oral doses of 1.2 and 2 gm. (table 3, typical of ten similar experiments). In all cases absorption was rapid, peak plasma levels being achieved in one to two

hours. The magnitude of this peak, however, was small and showed considerable variation for different subjects. The drug disappeared from the plasma so rapidly that only traces were found after five hours. Methemoglobin formation was small and considerably lower than after similar doses of acetanilide (1).

Acetophenetidin and its Transformation Products Found in Urine. Information concerning the transformation products of acetophenetidin was obtained from the examination of the urine of six subjects given 1 and 2 gm. of the drug orally (table 4). The urines were collected for the succeeding 24 hours and examined for the drug and its transformation products. Negligible quantities of the parent drug or its metabolites were excreted after 24 hours. Approximately 0.2 per cent of the administered acetophenetidin was excreted unchanged, 0.1 per cent as the deacetylated form, p-phenetidin, (p-ethoxy aniline), 3.5 per cent as the deethylated form, N-acetyl p-aminophenol, and the major portion, about 74 per

TABLE 4

The metabolic fate of acetophenetidin in man

Recovery of acetophenetidin and its metabolic products from the urine of subjects given single oral doses of acetophenetidin.

The urine was collected over a period of 24 hours. The proportion of the various metabolites is expressed in percentage of the amount of acetophenetidin administered.

DRUG ADMINISTERED	ACETOPHENETIDIN	P-PHENETIDIN	N-ACETYL P-AMINOPHENOL	CONJUGATED N-ACETYL P-AMINOPHENOL
gm.	per cent	per cent	per cent	per cent
1.0	0.17	0.12	3.5	82
1.2	0.36	0.12	4.7	65
2.0	0.15	0.04	3.1	80
2.0	0.30	0.15	3.6	81
2.0	0.23	0.03	2.6	81
2.0	0.23	0.13	3.9	57

cent, as conjugated N-acetyl p-aminophenol.⁸ The nature of the conjugation at the hydroxyl group was not investigated but by analogy with the conjugated N-acetyl p-aminophenol formed from acetanilide it was presumably a mixture of the sulfate and glucuronate (7). That the conjugated p-aminophenol was also acetylated was surmised from the absence of free amino groups in the urine other than that due to the small amount of p-phenetidin present. Free p-aminophenol was not detected in the urine.

Acetophenetidin and its Transformation Products Found in Plasma. Plasma concentrations of acetophenetidin and its transformation products, were measured in subjects after single oral doses of 1.2 gm. of acetophenetidin (fig. 1, typical of seven similar experiments). The levels of the parent drug were low and declined rapidly while those of its derived product, N-acetyl p-aminophenol quickly rose to considerable levels, suggesting that acetophenetidin was quickly deethylated to this compound. The levels of N-acetyl p-aminophenol persisted when aceto-

⁸ Conjugated N-acetyl p-aminophenol is total conjugated p-aminophenol less the N-acetyl p-aminophenol.

phenetidin had already declined to negligible proportions. Conjugated N-acetyl p-aminophenol, formed from N-acetyl p-aminophenol, remained elevated beyond

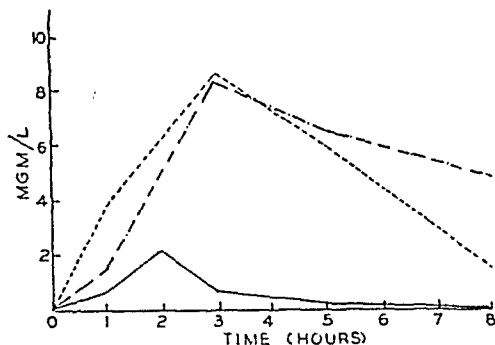


FIG. 1. Plasma levels of acetophenetidin (solid line) and its metabolites, N-acetyl p-aminophenol (dashed line), and conjugated N-acetyl p-aminophenol (dash-dot line) after the oral administration of 1.2 gm. of acetophenetidin to man.

TABLE 5

Distribution of acetophenetidin, p-phenetidin and N-acetyl p-aminophenol in dog tissues

The dog received 2.7 gm. of acetophenetidin orally. The tissues were examined two hours after the administration of the drug.

TISSUE	CONCENTRATION IN TISSUES			RATIO CONC. IN TISSUE WATER CONC. IN PLASMA WATER		
	Aceto- phenetidin	p-phenet- idin	N-acetyl p-amino- phenol	Aceto- phenetidin	p-phenet- idin	N-acetyl p-amino- phenol
	mgm./kgm.	mgm./kgm.	mgm./kgm.			
Plasma.....	57	12.3	96			
Red Cells.....	60	13.5	96	1.60	1.70	1.50
C.S.F.....	36	8.5	49	0.59	0.61	0.48
Liver.....	91	50.5	99	2.10	5.45	1.32
Kidney.....	67	26.5	104	1.40	2.62	1.29
Heart.....	56	19.2	70	1.21	1.89	1.00
Spleen.....	68	13.5	80	1.44	1.37	1.00
Lung.....	70	20.2	88	1.47	1.97	1.11
Brain.....	77	16.5	82	1.57	1.61	1.00
Muscle.....	48	17.1	69	1.05	1.74	0.90

the time that N-acetyl p-aminophenol had declined to low levels. Neither p-phenetidin nor free p-aminophenol could be detected in plasma.

Acetophenetidin and its Transformation Products Found in Tissues. The distribution of acetophenetidin and its transformation products, p-phenetidin and

N-acetyl p-aminophenol, was examined in representative tissues of a dog given 2.7 gm. of acetophenetidin orally. Two hours after drug administration the animal was killed by an intravenous injection of air and the tissues were sampled immediately. The tissue water content was determined by drying to constant weight at 95–100° C. The tissue concentration of the various compounds was measured and calculated in terms of tissue water (table 5). N-acetyl p-aminophenol was found to be distributed fairly evenly in terms of body water, while acetophenetidin and p-phenetidin were concentrated to a considerable degree in most tissues. All three compounds were concentrated to some degree in red blood cells and there was some hindrance to their free passage into the cerebrospinal fluid.

The extent to which the compounds were bound to plasma proteins was determined by dialysis against isotonic phosphate buffer of pH 7.4 at 37° C.

TABLE 6

Plasma levels of N-acetyl p-aminophenol and conjugated N-acetyl p-aminophenol after the oral administration of 1 gram N-acetyl p-aminophenol to man

TIME	SUBJECT A		SUBJECT B	
	N-acetyl p-aminophenol	Conjugated N-acetyl p-aminophenol	N-acetyl p-aminophenol	Conjugated N-acetyl p-aminophenol
hours	mgm./L.	mgm./L.	mgm./L.	mgm./L.
1	2.9	2.8	5.8	4.2
2	8.2	9.8	10.9	11.0
3	4.6	13.4	7.5	12.0
5	2.6	8.2	2.9	8.3
8	0.0	2.8	0.0	5.0

Visking membranes were used for the dialysis bags. Acetophenetidin and p-phenetidin were bound to the extent of approximately 30 per cent, N-acetyl p-aminophenol to the extent of approximately 25 per cent at the plasma levels occurring in the above dog experiment.

Fate of the Metabolites of Acetophenetidin in the Body. Previous work with N-acetyl p-aminophenol showed that approximately 85 per cent of the orally administered compound was recovered in the urine in a conjugated form while approximately 4 per cent was excreted unchanged (1). The probable role of N-acetyl p-aminophenol in the analgesic action of acetophenetidin prompted a further study of its physiological disposition. Subjects were given 1.0 gm. of N-acetyl p-aminophenol orally and plasma levels of the compound and its conjugated form were determined at various time intervals. The drug was absorbed rapidly, peak levels being achieved in one to two hours (table 6). Negligible concentrations of the drug persisted after eight hours. Peak levels of the conjugate were achieved somewhat later and the levels persisted longer. The administration of N-acetyl p-aminophenol was not attended by the formation of methemoglobin.

Because of the role it plays in the toxicity of acetophenetidin, the fate of

p-phenetidin was studied in dogs. Twenty-four mgm./kgm. of p-phenetidin was administered orally to two dogs and urine was collected for 48 hours subsequently. Approximately 2 per cent of the parent drug was excreted unchanged and 40 per cent as conjugated p-aminophenol. The fate of the remainder of the p-phenetidin was not determined. The conjugated p-aminophenol in the urine was not acetylated since it gave a reaction for a free amino group, a fact which is in accord with the observation that the dog does not acetylate amino groups. By analogy with the known fate of aniline which forms hydroxy conjugated p-aminophenol in the dog but conjugated N-acetyl p-aminophenol in man, it is probable that acetylation would occur in the human (1).

The Role of p-Phenetidin in the Formation of Methemoglobin. Acetophenetidin is reputed to be less toxic than acetanilide, causing less methemoglobin and anemia (8). This is consistent with the smaller degree of deacetylation to an aromatic amine. In fact p-phenetidin could not be detected in plasma after the ingestion of therapeutic doses of acetophenetidin in man. However a small amount of p-phenetidin was found in urine, indicating that some deacetylation

TABLE 7

Correlation of p-phenetidin and methemoglobin levels in the blood after the administration of p-phenetidin and acetophenetidin to dogs

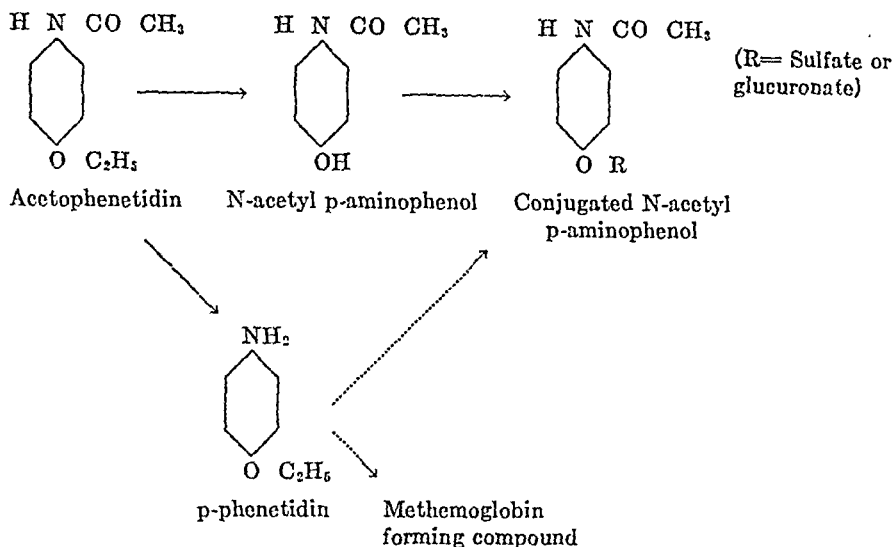
DOG	SUBSTANCE ADMINISTERED	AMOUNT ADMINISTERED	PLASMA p-PHENETIDIN MAXIMUM LEVELS	MAXIMUM METHEMOGLOBIN
		mgm./kgm.	mgm./L.	per cent
1	Acetophenetidin	75	4.3	32
2	Acetophenetidin	75	5.2	33
1	p-Phenetidin	12	5.0	45
2	p-Phenetidin	12	6.6	47

had occurred. Small amounts of methemoglobin were found after the oral administration of acetophenetidin. Methemoglobin was also found after the administration of small amounts of p-phenetidin to dogs. This suggested that the methemoglobin in the blood after the administration of acetophenetidin might have been formed as the result of amounts of p-phenetidin too small to be identified chemically. The evidence for this was that the amount of methemoglobin in the dog was correlated with the plasma p-phenetidin concentration subsequent to the administration of large doses of either acetophenetidin or p-phenetidin (table 7). Whole blood incubated for 2 hours at 37° C. with either drug at concentrations of 200 microgm. per cc. showed no accumulation of methemoglobin. It was concluded from this that the hemoglobin in the body was not oxidized to methemoglobin directly by p-phenetidin but by some product derived from it in the organism.

As in the case of acetanilide, the nature of the actual methemoglobin forming compound is not known. It is not p-aminophenol since this compound was not present in detectable amounts in the blood or urine. Neither is it N-acetyl p-aminophenol because no methemoglobin is formed after its administration.

The methemoglobin forming compound is derived solely from the formed p-phenetidin.

Discussion. The following route of metabolism of acetophenetidin in man is suggested by the observations described in this paper.



The main route of metabolism appears to involve two serial steps. The first is rapid deethylation at the ethoxy group to form N-acetyl p-aminophenol, the same metabolite that results from acetanilide by oxidation (1). The second step is conjugation of N-acetyl p-aminophenol at the hydroxyl group presumably with sulfuric or glucuronic acid. A small part of the acetophenetidin deacetylates to yield p-phenetidin. This compound is in turn deethylated and presumably converted in part to N-acetyl p-aminophenol. p-Phenetidin also appears to be the precursor of the substance which is responsible for the formation of methemoglobin.

N-acetyl p-aminophenol has long been known as an active antipyretic in man (9). Previous work has suggested that it is also an active analgesic (10). The rapidity with which it forms in the body from acetophenetidin suggests that the activity of the parent compound is directed through N-acetyl p-aminophenol.

SUMMARY

1. Methods are described for the determination of acetophenetidin and p-phenetidin in biological fluids and tissues.

2. The route of metabolism of acetophenetidin in man is as follows: the major fraction of the drug is rapidly deethylated to N-acetyl p-aminophenol; this compound is excreted in a conjugated form; a minor fraction deacetylates to form p-phenetidin; this compound was shown to be the precursor of the substance which oxidizes hemoglobin to methemoglobin. The analgesic and antipyretic action of acetophenetidin is exerted mainly through N-acetyl p-aminophenol which is an active analgesic and antipyretic.

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REFERENCES

1. BRODIE, B. B., AND AXELROD, J.: *THIS JOURNAL*, 94: 29, 1948.
2. WILLIAMS, R. T.: *Detoxication Mechanisms*, p. 149, John Wiley & Sons, New York, 1947.
3. BRODIE, B. B., UDENFRIEND, S., AND BAER, J. C.: *J. Biol. Chem.*, 168: 299, 1947.
4. BRODIE, B. B., UDENFRIEND, S., AND TAGGART, J. V.: *J. Biol. Chem.*, 168: 327, 1947.
5. BRODIE, B. B., AND AXELROD, J.: *THIS JOURNAL*, 94: 22, 1948.
6. EVELYN, K. A., AND MALLOY, H. T.: *J. Biol. Chem.*, 126: 655, 1938.
7. LESTER, A. D., AND GREENBERG, I. A.: *THIS JOURNAL*, 90: 68, 1947.
8. GOODMAN, L., AND GILMAN, A.: *The Pharmacological Basis of Therapeutics*, p. 244, The Macmillan Co., New York, 1941.
9. HEFFTER, A.: *Handbuch der Exper. Pharmacol.*, p. 1070, Julius Springer, Berlin, 1923.
10. FLINN, F. B., AND BRODIE, B. B.: *THIS JOURNAL*, 94: 76, 1948.

and even more of the *l* isomer of desoxyephedrine. The hydrogenated compounds are much less active in raising the blood pressure than would be expected from the animal data, but the relationship between the optical isomers remains the same; i.e., the *l* isomers are more active than the *d*, and the *N*-methyl derivatives are less active than the unsubstituted compounds.

As an approximation of the duration of action, the time that the blood pressure was elevated over one-half the total increase in pressure was used (10, 12). The average "50 per cent duration times" are given in table 1. The differences in duration in the dog experiments are very small and may not be significant, but the trend is for the unsubstituted compounds to be longer in duration than the *N*-methyl compounds, and the aromatic compounds to be longer in duration than the cyclohexyl compounds with the *d* isomers of the aromatic compounds longer acting than the *l*, but the *l* isomers of the cyclohexyl derivatives longer than the *d*. The durations of action of the agents in man are not significantly different at the dose levels that produce comparable rises in blood pressure. The agents start to elevate the blood pressure 45 to 75 minutes after ingestion and the elevation lasts an additional 90 to 135 minutes.

Even the rather large doses of the cyclohexyl compounds given to man did not produce any of the euphoria or talkativeness always observed with the doses of the phenyl compounds given. The 17–23 mm. rise in blood pressure used as an index was accompanied by a comparable rise in diastolic blood pressure and a concomitant decrease in pulse rate, but there was no incidence of dry mouth, palpitation, headache, numbness of extremities, or exaggerated pilomotor response in the 24 individuals used as test subjects. In seven other individuals of the original 40 that exhibited rises in systolic blood pressure greater than 30 mm. Hg following the administration of 12.5 mgm. *d*-amphetamine, some or all of these effects were noted.

The results for the racemic or *dl*-mixtures of the compounds are intermediate between the results for the separate isomers.

DISCUSSION. Alles (6) found *d* and *l*-amphetamine to be almost equipotent in etherized rabbits although the *d* isomer was more active in man. Similarly, we found these compounds to differ very little in barbitalized dogs, although the *d* isomer was more active in unanesthetized dogs. These results are the opposite of those of Swanson *et al.* (7) which were obtained in pithed dogs. Lands *et al.* (8) suggested that a similar discrepancy between the results of the two groups for the desoxyephedrine isomers might be explained on the basis of a greater effect of the *d* isomer on the central vasomotor center.

Although the rotation of plane polarized light by the *l* or more active cyclohexyl compounds is opposite to that of the more active compounds in the aromatic group, the more active agents presumably have the same configuration about the asymmetric carbon atom, since the levorotatory cyclohexylisopropylamine is synthesized by the reduction of the dextrorotatory amphetamine (9). Alles (6) has pointed out that Leithe's work indicates that dextrorotatory amphetamine has the same configuration as *l*-ephedrine. Consequently, the more active agent in all of these pairs of compounds may be presumed to have the same spatial

configuration about the asymmetric carbon atom, and the relative positions of the ring and the nitrogen atom in space would be the same. The greater activity might be due to a better fit into the molecular architecture of the activating area in sympathetic end organs which are designed to be activated by sympathins structurally related to *l*-epinephrine.

Although Gunn and Gurd (13) found *dl*-cyclohexylisopropylamine to have some central nervous system stimulant activity in groups of mice, no obvious indications of this effect were observed in man with doses of any of the cyclohexyl compounds that were as much as ten times that required to give an obvious central nervous stimulating effect by *d*-amphetamine.

The effect of the transition from intravenous administration in dogs to oral administration in man is pronounced, since the least active cyclohexyl compound is almost one-third as active as the most active phenyl compound in the dog, but the spread has increased until the least active compound is only one-tenth as active as the most active one in man.

SUMMARY

d-Amphetamine sulfate is more active than *l*-amphetamine sulfate in raising the blood pressure of barbitalized and unanesthetized dogs. Both of these isomers are more active than the corresponding desoxyephedrine hydrochlorides, although *d*-desoxyephedrine is more active than *l*. Conversely, *l*-cyclohexylisopropylamine hydrochloride is more active than *d*, and they both are slightly less active than the corresponding amphetamine isomers. The cyclohexylisopropylmethylamine hydrochlorides are the least active of all, although the *l* isomer is more active than the *d* isomer.

On oral administration in man, *d*-amphetamine sulfate is the most active agent in this group in raising the blood pressure, with the *l* isomer of amphetamine and *d*-desoxyephedrine about one-half as active, *l*-desoxyephedrine about one-third, the cyclohexylisopropylamines one-sixth to one-eighth, and the cyclohexylisopropylmethylamines only one-eighth to one-tenth as active. The amphetamine and desoxyephedrine isomers produce obvious central nervous system stimulation in man while the cyclohexylisopropyl and cyclohexylisopropylmethylamines do not.

REFERENCES

1. BARGER, G., AND DALE, H. H.: *J. Physiol.*, **41**: 19, 1910-1911.
2. CUSHNY, A. R.: *J. Physiol.*, **33**: 259, 1909.
3. CHEN, K. K., WU, C. K., AND HENRIKSEN, E.: *This Journal*, **36**: 363, 1929.
4. TAINTER, M. L., AND STOCKTON, A. B.: *Am. J. Med. Sci.*, **185**: 832, 1933.
5. SCHAUAMANN, O.: *Arch. f. exper. Path. u. Pharmacol.*, **181**: 137, 1936.
6. ALLES, G. A.: *Univ. Calif. Pub. in Pharmacol.*, **1**: 129, 1939.
7. SWANSON, E. E., SCOTT, C. C., LEE, H. M., AND CHEN, K. K.: *This Journal*, **79**: 329, 1943.
8. LANDS, A. M., NASH, V. L., GRANGER, H. R., AND DERTINGER, R. L.: *This Journal*, **89**: 382, 1947.
9. ZENITZ, B. L., MACKS, E. B., AND MOORE, M. I.: *Jour. Am. Chem. Soc.*, **69**: 1117, 1947.
10. MARSH, D. F.: *This Journal*, **93**: 338, 1948.
11. LAMBERT, E. H., AND WOOD, E. H.: *Proc. Soc. Exper. Biol. and Med.*, **64**: 186, 1947.
12. WINDER, C. V., ANDERSON, M. M., AND PARKE, H. C.: *This Journal*, **93**: 63, 1948.
13. GUNN, J. A., AND GURD, M. R.: *J. Physiol.*, **97**: 453, 1940.

THE ASSAY OF CURARE BY THE RABBIT "HEAD-DROP" METHOD¹

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The name curare has been applied to potent preparations obtained by aqueous extractions of certain South American plants. Typical curare activity, as exemplified by the action of extracts of *Chondodendron tomentosum*, is characterized by a quantitative interference with the transmission of nerve impulses to the effectors in voluntary muscle. The evident result, therefore, of parenteral administration of curare is the relaxation of skeletal musculature. It is this property which makes curare and its alkaloids useful to the anesthetist, surgeon, psychiatrist and neurologist. The availability of stable, purified, standardized preparations of curare for clinical use has been dependent largely upon the development of an accurate, reproducible biological method for their potency determination.

The purpose of this paper is to present in detail the head-drop assay method used in these laboratories over a period of nine years for the determination of the potency of various curare extracts and alkaloids, and to describe briefly other assay procedures studied prior to its development. Other methods have been reported within this interim for the assay of curare or curare-like preparations, which have utilized the typical effect of curare on intact animals (8-21), on isolated nerve-muscle (21-25) and *in situ* nerve-muscle (13, 14, 15, 20) preparations, on the acetylcholine contraction of isolated skeletal muscles (19, 26, 27), and in morphinized animals (28).

EXPERIMENTAL. In selecting a suitable method for the assay of curare preparations, several procedures have been investigated and evaluated. The method of finding the minimum dose of curare required to produce paralysis in frogs following injection into the ventral lymph sac was found impractical because of the very large number of animals necessary to obtain a precise evaluation of relative activity of test sample and standard preparation. Determination of the lethal dose by subcutaneous injection into mice and other animals has also been used as a method for standardizing curare extracts. Our results when using the subcutaneous LD₅₀ for evaluation of potency indicated that the same objections apply to it as to the frog-paralysis test. Skinner and Young (17) have recently reported a subcutaneous method for determination of the 50 per cent effective dose, utilizing a slanting, rotating cylinder from which paralyzed mice fall. They reported greater precision than we were able to obtain with the subcutaneous LD₅₀ procedure given above.

¹ Part of the material in this paper was presented before the American Society for Pharmacology and Experimental Therapeutics, Federation meetings, Atlantic City, March 17, 1948. See Fed. Proc., 7: 261, 1948.

The basic method was described briefly by Bennett (1), Holaday (2) and Dutcher (3) and has been used in various modifications by several investigators (4-16).

The determination of the intravenous LD_{50} (50 per cent anoxic fatal dose) in three species was investigated as another approach to this problem. Although this procedure proved fairly accurate in the rabbit, it was considered too costly for routine assays. In comparison with the results obtained in rabbits, those in mice and particularly those in rats showed much greater variation in response to a single rapid injection. This was due in part to the early appearance in the rodent of an anoxic condition with convulsive effects before all the respiratory muscles were paralyzed. However, contrary to the findings of Cohnberg (29), it was found that such convulsions could be largely obviated or that rats showing such movements became relatively quiet when positive pressure artificial respiration was given through a tracheal cannula.

Concurrently with these studies the curare effect on local muscle groups was investigated as a possible assay procedure. As the result of many trials with various mammalian nerve-muscle preparations *in situ* and with amphibian preparations *in situ* and after isolation, a method utilizing the isolated frog gastrocnemius muscle was evolved. It was found that this muscle, when suspended in a bath of modified Ringer's solution, would contract upon addition of acetylcholine to the bathing fluid. This contraction, recorded isometrically, was partially or completely inhibited by the prior addition of curare extract to the bath. The degree of inhibition was quantitatively related to the concentration of curare in the bathing fluid by logarithmic function. This method was extremely accurate, giving very high dose-response correlation (0.999), and was used extensively for comparison of the activity of fractions obtained in the purification of various curare extracts. Unfortunately, it was found that some curare preparations, even from the same source, purified by the same procedure and standardized by the frog gastrocnemius method, showed widely different activities when used in humans.

Development of the Rabbit Head-drop Assay

In the early study of the effect of curare in the intact, unanesthetized animal, it was decided that, of all the muscle groups successively affected, the relaxation of those supporting the head appeared most suitable to use as the endpoint of the assay (head-drop). Preliminary investigations of the parenteral modes of administration of curare showed that the intravenous route gave the most constant and reproducible results since the curare effect was fairly rapid in onset and of comparatively short duration. The rabbit was first chosen as the experimental animal because of the ease of making repeated intravenous injections in this species. Later when the assay method, utilizing slow intravenous titration to head-drop, had been better established, a study of the variation of the daily head-drop dose of curare by this method revealed that the rabbit yielded more constant results than the rat or the mouse. Even though the endpoint was found to be fairly sharp and quite constant from day to day in the dog, cat and monkey, these animals were not used for routine testing because they were more difficult to handle, more expensive and less easily obtained than rabbits.

Rate of Administration: In a study of the effect of rate of injection on the

sharpness of the head-drop endpoint, on the head-drop dose and on recovery time, several series of experiments in rabbits were conducted. When the material was injected too slowly the administration rate so closely approached the elimination rate that the endpoint either was not reached or was achieved only after administration of relatively large amounts of the drug over an unduly prolonged period. On the other hand when the administration was too rapid the full effect of the amount of curare given was not obtained at the time head-drop was reached, and later an over-effect was produced as evidenced by prolonged curarization and increased incidence of respiratory paralysis. Further, and of more importance, the rapid progression of curarization from the initial signs of relaxation of the neck muscles to complete respiratory paralysis made it impossible to differentiate clearly the head-drop endpoint.

When curare was injected intravenously at an intermediate rate into a normal rabbit, a gradually developing relaxation of the muscles of the back and neck resulted and the endpoint was sharply defined and easily determined. (For description of endpoint see "The Head-drop Endpoint" under the Rabbit Head-drop Assay Procedure.) Photographs from these laboratories showing progressive stages of curarization in the rabbit including the head-drop endpoint were published by Bennett (1). A 2 unit per cc. extract of the reference standard curare powder, given at a rate of 0.1 cc. every fifteen seconds, resulted in a satisfactory endpoint within three to eight minutes from the beginning of the intravenous injection. (For definition of unit see "Preparation of Solutions" under the Rabbit Head-drop Assay Procedure.)

The data presented in table 1 show the effect of change in injection rate. A concentration of 2 units per cc. of a single solution was used throughout the series. Prior to use in these experiments each rabbit had been given a head-drop dose of curare at a control rate of 0.1 cc. each fifteen seconds on at least two consecutive days (see section on "Necessity for Preliminary Injection"). Each animal in each group then received on successive days the same solution at two different rates, namely, at the control rate and at an experimental rate of either one-fifth, one-half, two or four times the control rate. For each group of animals the arithmetic mean of the injection time, head-drop dose and recovery time appear in the first part of table 1, with the standard errors for the first two of these values which were calculated from the deviations of the results for individual animals in each group. The figures which are given in the last three columns of the table were calculated by determining the ratios of the experimental and control values for each animal and obtaining the geometric mean of these ratios for each group. Since the standard errors of the mean in each of these last three columns were calculated from the deviations of the ratios, i.e., paired injections in the same animals, they are, of course, lower than those appearing in the preceding columns.

When the experimental injection was given most rapidly, i.e., at four times the control rate (Group A, table 1), the average experimental head-drop dose and recovery time were markedly increased, and 80 per cent of the animals showed respiratory paralysis. When the experimental injection rate was twice the control

rate (Group B), the average experimental head-drop dose was not significantly different from that of the control, but the average recovery time was significantly longer. Although no respiratory paralysis was observed in experimental Group B, it was found in further study of this rate of injection that the percentage of animals requiring artificial respiration was so high as to be impractical for routine assays. Furthermore, the dose increment at this rate was about 8 per cent of the total dose, whereas with the control rate it was about 4 per cent, the latter giving a more critical determination of the endpoint.

A decrease in the rate of injection to one-half the control rate (Group C) caused no significant change in either the average experimental head-drop dose or the average recovery time. However, since about thirteen minutes were required to reach the endpoint compared with about six minutes at the control

TABLE 1

The Effect of Various Injection Rates by Crossover Pattern on the Average Head-drop Dose and Recovery Time in Rabbits

(A single 2 unit per cc. curare solution injected intravenously)

	NO. OF BABBITS	NO. OF INJECTIONS	INJECTION RATE, CC./15 SEC.	AVERAGE TIME FOR HEAD-DROP, MINUTES \pm % S.E.	AVERAGE DOSE FOR HEAD-DROP, UNITS PER ANIMAL \pm % S.E.	AVERAGE RECOVERY TIME AFTER HEAD-DROP, MINUTES	AVERAGE OF RATIOS FOR INDIVIDUAL ANIMALS: $\frac{\text{EXPERIMENTAL}}{\text{CONTROL}} \pm$ % S.E.*		
							Injection Time	Head-drop Dose	Recovery Time
(A) Exptl. rate	10	10	0.4	1.95 \pm 5.0%	6.24 \pm 5.0%	25.7**	0.32 \pm 3.6%	1.27 \pm 3.6%	3.08 \pm 12.7%
Control rate		10	0.1	6.18 \pm 6.4%	4.94 \pm 6.5%	8.6			
(B) Exptl. rate	11	11	0.2	3.52 \pm 4.1%	5.61 \pm 3.7%	17.8	0.53 \pm 0.8%	1.09 \pm 0.8%	1.59 \pm 9.2%
Control rate		11	0.1	6.44 \pm 4.3%	5.16 \pm 3.9%	10.7			
(C) Exptl. rate	10	10	0.05	13.2 \pm 6.8%	5.23 \pm 6.8%	10.0	2.18 \pm 2.0%	1.09 \pm 2.1%	1.06 \pm 9.9%
Control rate		10	0.1	6.03 \pm 5.9%	4.82 \pm 5.9%	9.0			
(D) Exptl. rate	10	10	0.02	43.9 \pm 9.2%	7.02 \pm 9.2%	11.0	7.12 \pm 4.5%	1.42 \pm 4.6%	1.23 \pm 6.6%
Control rate		10	0.1	6.05 \pm 6.5%	4.81 \pm 6.5%	8.9			

$$* \% \text{S.E.} = \frac{V}{\sqrt{N-1}} \text{ or } \frac{\sigma^2}{\bar{x}} \times 100$$

** Eight out of ten animals showed respiratory paralysis.

rate, the test would be excessively time-consuming if carried out routinely at this rate of injection. When the injection was made at one-fifth the control rate (Group D), the average time required to reach head-drop was further extended. The 42 per cent increase in the average head-drop dose at this very slow rate was due in part to elimination of curare and in part to greater saturation of the tissues, the latter being indicated by a significant increase in recovery time.

Since the standard and test samples are injected at the same rate in the assay of curare by the crossover method, valid assays can be conducted by using injection rates within the range of 0.05 to 0.2 cc. of a 2 unit per cc. solution every fifteen seconds. For reasons given above, the control rate, 0.1 cc. of a 2 unit per cc. concentration given every fifteen seconds, was chosen for routine assays. It was found that a satisfactory endpoint could also be reached by the rapid injection of a fixed percentage of the expected head-drop dose at the start of the test, followed by titration to head-drop at a slower rate. Since the head-drop dose

varies widely from rabbit to rabbit and may change somewhat throughout a series of daily administrations in the same rabbit, it was necessary to determine this dose periodically for each rabbit at the control rate in order that equivalent percentages of the same would be given as the initial rapid injection. For reasons of simplicity, this method was abandoned.

Effect of Body Weight: To ascertain whether the dose necessary to reach the endpoint of the test was best expressed as dose per kgm. of body weight or as dose per rabbit, an analysis of the correlation between dose and body weight was made. From the earlier data a group of 80 rabbits² was selected at random for this purpose. Each rabbit had received at least five head-drop injections at the rate of 0.1 cc. of a 2 unit per cc. solution each fifteen seconds, the average number of daily injections per rabbit being 9.7. Excluding the preliminary doses for reasons explained in the section on "Necessity for Preliminary Injections", the average weight "a" of each rabbit in kgm. and the average total dose "b" necessary to cause head-drop in each rabbit were recorded. The correlation between "a" and "b" was then calculated using the following equation:

$$r = \frac{\overline{ab} - \bar{a} \cdot \bar{b}}{\sigma_a \cdot \sigma_b}$$

The application of this formula gave a calculated value for "r" of 0.600 ± 0.057 standard error. This degree of correlation is not sufficient to contribute significantly to further reduction of the low standard error obtained in the crossover pattern which design practically eliminates any effect of differences in body weight upon the calculated potency.

Necessity for Preliminary Injections: In order to determine whether the first few daily head-drop doses given to a rabbit were significantly different from or varied more than subsequent doses, an analysis was made of the variation in daily dose of curare necessary to cause head-drop. The data from 49 rabbits were selected at random; these rabbits received a minimum of eight head-drop doses each (an average of 14.1) at a rate of 0.1 cc. of a 2 unit per cc. solution each fifteen seconds. As a basis for comparison the average dose for each rabbit was calculated, the first three doses being excluded from this average by preliminary inspection of the data. The ratio of the first, second, and third to the average dose was calculated for each rabbit and the geometric means of these ratios are given in table 2. From the P values it may be seen that the first and second doses were significantly lower than the average dose, whereas this was not true of the third dose.

The coefficient of variation for the ratio of the first, second and third to the average dose was 24, 17 and 16 per cent, respectively, indicating that between the first and second doses there was an appreciable decrease in the variation between animals. Therefore at least two daily preliminary head-drop injections should be given to a rabbit before it is used in the assay. It is also unwise to use a

² Here and elsewhere in the following experimental sections, typical data chosen at random from hundreds of head-drop assays were calculated, using sufficient data, however, to render the conclusions valid without making computation unduly burdensome.

rabbit for assay after a rest period of five days or more without at least two such preliminary curarizations.

Number of Head-drop Injections per Day: Since the symptoms of curarization in rabbits disappear within about ten minutes, a study was made to determine whether each animal after two or three preliminary daily injections could receive more than one titration to head-drop in a single day without a significant decrease in the head-drop dose. Lacking a suitable method for estimation of curare blood

TABLE 2
Relation of First, Second, and Third to the Average Daily Head-drop Dose*
(49 Rabbits)

	GEOMETRIC MEAN OF RATIO \pm % S.E.**	DEVIATION FROM UNITY \pm S.E.	RATIO DEVIATION TO S.E. (INDEX OF SIGNIFICANCE)	P
First				
Average	0.853 \pm 3.40%	0.147 \pm 0.030	4.9	<0.001
Second				
Average	0.899 \pm 2.42%	0.101 \pm 0.022	4.6	<0.001
Third				
Average	0.972 \pm 2.28%	0.023 \pm 0.022	1.3	0.2

* Average based on fourth to last daily dose inclusive.

** % S.E. = $\frac{V}{\sqrt{N-1}}$ or $\frac{\sigma_x}{\bar{x}} \times 100$.

TABLE 3

Percentage of Original Head-drop Dose of Curare "Eliminated" in One to Six Hours
(Determined by a Second Titration to Head-drop)

NUMBER OF RABBITS	HOURS BETWEEN 1ST AND 2ND TITRATIONS	SECOND HEAD-DROP DOSE FIRST HEAD-DROP DOSE $\times 100$
4	1.0	63.6
5	2.0	76.1
10	3.5	87.6
31	4.5	92.0
3	6.0	100.0

levels, the procedure consisted of following an initial titration to head-drop with a second titration at intervals of 1, 2, 3.5, 4.5 or 6 hours in the respective groups of rabbits. It may be seen in table 3 that significant reduction in the second head-drop dose occurred even at 4.5 hours following the first dose. Thus it was considered inadvisable to use rabbits for head-drop evaluation of curare preparations more than once daily.

The Rabbit Head-drop Assay Procedure

Having demonstrated in the rabbit head-drop assay that (a) no advantage accrued from dosage based on body weight, (b) head-drop dosage became sufficiently constant after the

second daily injection, and (c) injections should not be repeated in less than six hours—the following procedure was adopted.

Animals Employed: Healthy rabbits which weigh from 2.0 to 3.5 kgm. and have received at least two head-drop doses within the previous seven days are used for the assay. The rabbits are fed an adequate diet of complete pellets³, and food and water are not withdrawn before the assay. The animals may be used daily but not more than one injection should be given each day.

Preparation of Solutions: The standard solution is prepared at exactly 2 units per cc. The reference standard used throughout most of the investigations reported herein and until recently was a particular stable desiccated curare powder stored to prevent change in moisture content. A solution was prepared by (a) adding 2.0 mgm. of this powder per cc. of water, (b) shaking the mixture for 30 minutes at room temperature and (c) removing the slight amount of insoluble matter by centrifugation. To 0.5 cc. of this solution was arbitrarily assigned the potency of one unit. Isolation of crystalline *d*-tubocurarine chloride, first from "tube" curare by King (30) and later from *Chondodendron tomentosum* by Wintersteiner and Dutcher (31) has, however, made it possible to express one unit as equivalent in curare activity to 0.155 mgm.⁴ of a particular sample of recrystallized *d*-tubocurarine chloride pentahydrate (11.46 per cent moisture) accurately assayed against the original reference standard. This standard is prepared for injection at a concentration of 0.310 mgm. per cc.

The potency of the test solution to be injected should be adjusted to within ± 10 per cent of that of the standard (2 units per cc.). If the potency of the test solution is unknown, the adjustment to approximately 2 units per cc. may be based upon comparison in one to three rabbits of the head-drop volume of a trial dilution of the test sample with their previously determined head-drop volumes of a reference solution adjusted to 2.0 units per cc.

The Head-drop Endpoint: The endpoint of the assay, head-drop, is the precise relaxation state when the animal's head falls to the board and cannot be raised or turned in response to a light tap on the animal's back. This stimulus is necessary in order to determine whether the true endpoint has been reached because the lowering of the head may be in part voluntary, especially in animals used repeatedly.

The Crossover Pattern: The assay is conducted by the crossover pattern in order to minimize the influence of possible day-to-day changes in the sensitivity of the group of animals.

³ Rockland Rabbit Ration or Purina Rabbit Chow Checkers.

⁴ For practical purposes the unit as here defined may be expressed to the second digit (i.e., 0.15 mgm.), as given in New and Nonofficial Remedies—1948, page 221. However, we wish to report that we have found variations in the potency in units per mgm. of crystalline *d*-tubocurarine chloride as prepared by different manufacturers. Dr. Dutcher of the Division of Organic Chemistry, the Squibb Institute for Medical Research, found upon extensive purification of *d*-tubocurarine chloride, the details of which will be published elsewhere, that the impurities in the commercial preparations studied were of two types: (a) tertiary alkaloids which are inactive and (b) quaternary alkaloids which are much more active than *d*-tubocurarine. Whereas the two original materials used had 6.5 and 6.7 units per mgm. (pentahydrate) activity, the purified products had potencies ranging in single assays from about 5.8 to 6.2 units per mgm. (pentahydrate). Thus if the unit should be expressed in terms of the weight of pure alkaloid rather than in terms of the present satisfactory standard preparation, a change in the present weight equivalent for the unit would obviously be required. This emphasizes the importance of the biological assay of commercially prepared *d*-tubocurarine chloride and the necessity of a reference standard of defined unit activity. However, the standard need not be a compound of highest possible purity since the significant feature of biological control is the use of a reference material which provides biological activity of a definite type in a specified weight (the unit) of such standard. The unit defines neither the chemical purity of the reference material nor any particular degree of animal response.

The test sample is injected on the first day into at least eight rabbits and the reference standard into an equal number. On the following day the crossover is completed, i.e., those rabbits which were injected with the standard are given the test sample and *vice versa*. The rate of injection is 0.1 cc. every fifteen seconds regardless of body weight. Each 0.1 cc. quantity is injected as rapidly as possible by gravity from a 5.0 cc. microburette through a small bore, thick-walled rubber tubing attached to a needle inserted into the ear vein. The time between the injections is utilized to determine the degree of relaxation as curarization progresses. When the endpoint of the test is reached, the injected volume and the injection time are recorded. Occasionally an animal will need artificial respiration. A pressure-type respirator with head mask or a Drinker model for animals should be employed, since manual compression of the chest wall will expel little or no air from the lungs. West (32) pointed out in studies on curarine that this is due to the loss of tone of the curarized respiratory muscles, a resultant decrease of "negative" intrapleural pressure and thoracic volume, and possibly to some active or passive bronchoconstriction.

Calculation of Potency: The ratio of the volume of standard at 2 units per cc. to that of the test sample necessary to reach the endpoint is calculated for each rabbit. The geometric mean of these ratios together with its standard error is then calculated; this mean multiplied by the dilution of the test solution and by 2 (potency of standard in units per cc.) equals the potency of the undiluted sample in units per cc.

Accuracy of the Assay: To evaluate the accuracy of the assay, the following study was carried out to determine the coefficient of variation of the individual responses. The data from a group of 41 rabbits which had received a minimum of nine head-drop doses each (an average of 15.4) on different days were used. The first two doses were excluded from the calculations. The average coefficient of variation was ± 11.4 per cent of the mean dose (i.e., 90 per cent of the effective doses may be expected to fall within a range of ± 18.8 per cent of the mean).

In a crossover assay the accuracy of the ratio of the head-drop dose of standard to that of sample in the same animal is more important to ascertain than is the standard deviation of a single observation as calculated above. In order to evaluate the error of such a ratio, data were selected at random from the early and recent routine rabbit assays of regular manufacturing lots of curare preparations. The old data comprise a series of 320 injection pairs in 20 crossover assays ranging from 10 to 20 animals per assay, while the recent data represent 1700 injection pairs in 100 assays ranging from 8 to 23 animals per test. The average coefficients of variation for the 20 old assays and the 100 more recent ones were ± 12.0 and ± 4.4 per cent, respectively. It is evident from these values that, as experience with the method increased, less variation occurred, due in part to elimination of animals showing a high day-to-day variation in head-drop dosage and to a more critical adjustment (within ± 5.0 per cent) of the potency of the sample to that of the standard before the assay was begun.

Further evaluation of the accuracy of the assay may be obtained by an examination of the data presented in table 4. These results were obtained in 23 separate crossover assays of a sample of crystalline *d*-tubocurarine chloride, all assays being carried out against the same reference material, i.e., a 2 unit per cc. extract of the standard curare powder. In these tests which were conducted over a period of more than a year and were interspersed with regular control testing, no extra precautions were taken and the data are therefore representative of those obtained in the routine assay procedure.

The mean potency of this sample, calculated from the individual assay values weighted according to their respective standard errors, was 6.478 ± 0.0175 units per mgm. The data from these 23 assays involving 433 injection pairs has been subjected to analysis of variance to detect any significant "Between Assay Variance" (table 4). Examination of the Mean Square in the final column shows that the component of variance for "Between Assays" has no statistical significance even on this large number of animals, and to the extent it may exist, it is very small compared with the "Within Assay" Variance.

DISCUSSION. The assay method for curare as described herein is designed to give maximum accuracy under closely controlled conditions. Because of the variation in the amount of curare required for head-drop in different rabbits, the determination of potency by comparing the volume of sample with that of

TABLE 4

Results of 23 Rabbit Head-drop Crossover Assays of Crystalline d-Tubocurarine Chloride versus Standard Curare Powder

NO. OF INJECTION PAIRS	POTENCY UNITS PER MILLIGRAM	STANDARD ERROR AS PER CENT OF MEAN
9	6.343	± 2.25
15	6.349	± 1.92
28	6.362	± 1.34
17	6.372	± 1.15
18	6.389	± 1.11
24	6.392	± 1.60
23	6.422	± 1.51
15	6.425	± 1.30
22	6.425	± 1.06
19	6.425	± 0.89
16	6.438	± 1.22
23	6.455	± 1.04
26	6.461	± 1.13
16	6.478	± 1.29
18	6.504	± 1.83
17	6.527	± 1.37
16	6.527	± 1.84
22	6.537	± 1.09
30	6.540	± 1.11
24	6.626	± 1.15
15	6.636	± 1.70
10	6.672	± 1.26
20	6.701	± 2.01

Analysis of Variance

SOURCE OF VARIANCE	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARE OR VARIANCE	VARIANCE RATIO F
Between Assays.....	22	0.01855	0.000843	1.141*
Within Assays.....	410	0.30319	0.000739	1.0
Total.....	432	0.32174		

* F = 1.57 at P = .05.

F = 1.25 at P = .20.

standard in the crossover pattern, using slow intravenous titration, gave greater accuracy and precision than could be obtained by any method utilizing a per kgm. dose in different groups of rabbits. Some of the bioassays performed on other drugs in these laboratories have been compared with the rabbit head-drop assay as to the number of individual injections necessary to achieve the same low standard error which is obtained in this assay (± 2.0 per cent of the mean of five

injection pairs). The results of these calculations appear in table 5 and show that the curare assay requires about ten per cent as many individual injections as the next most precise method.

Up to the first of the year 1949 approximately 1100 samples of curare had been tested by this crossover assay method, involving a total of about 31,000 single daily injections into rabbits. A large number of these tests were conducted for the purpose of standardizing curare preparations for clinical trial. Many of these preparations, assayed and adjusted to 20 units per cc., were critically evaluated in humans by qualified clinical investigators and all were found to have uniform effectiveness when equal volumes were administered to the same patients. The reliability of the method is also shown by the fact that in no case was there a demonstrable difference in the clinical potency of other similar curare preparations so assayed and subsequently used routinely on a unit basis in thousands of humans, totalling more than 3,000,000 injections.

TABLE 5

Comparison of the Precision of Various Other Bioassays with the Rabbit Head-drop Method

ASSAY	METHOD	NUMBER OF ASSAYS CALCULATED	TOTAL NUMBER OF INJECTIONS MADE	NUMBER OF INJECTIONS FOR A ± 2.0 PER CENT STANDARD ERROR OF THE MEAN
Curare	Rabbit Head-drop	100	3400	10*
Digitalis	Cat Fatal Dose	20	407	106
Posterior Pituitary	Rooster Blood Pressure	26	316	569
Estrogens	Rat Vaginal Smear	12	12,900	2393
Insulin	Mouse Convulsion	10	57,677	7366

* Five injection pairs on a crossover test have been considered equivalent to ten single injections.

When applied to the evaluation of potency of new batches of crude curare powder or syrup, the head-drop method as described becomes useful in indicating qualitative differences in action as reflected by unusual responses of the animals. For example, analysis of the data from 1271 head-drop injections in rabbits has shown a 2.9 per cent incidence of respiratory paralysis with regular manufacturing lots of semi-purified and purified curare preparations. Any sample from new raw materials giving a higher incidence of respiratory paralysis than this may be suspected of a qualitative deviation from the norm. Similarly prolonged recovery time, peculiar progression of signs of curarization, evidence of central excitement, collapse, or other untoward reactions serve as indications that a sample representing unsatisfactory material is being injected.

The use of the rabbit head-drop assay as described for determination of potency is, however, limited to compounds having a fairly rapid onset of action and a duration of action not much shorter than that of *d*-tubocurarine chloride. Although certain alpha-substituted ethers of glycerol, which cause head-drop in animals by a central action, have onsets and durations of action comparable to those of peripherally acting *d*-tubocurarine chloride, it is felt that such com-

pounds having different sites and types of action cannot be compared quantitatively to *d*-tubocurarine chloride. Even with curare alkaloids or derivatives having similar pharmacodynamic action, widely different ratios of activity may be obtained upon administration to different species. On a mgm. basis *d*-tubocurarine dimethylether iodide was more than six times as effective as *d*-tubocurarine chloride in rabbits, but less than three times as effective clinically. In spite of such variations in potency, standardization of a new voluntary muscle relaxant may be confined to one species, such as the rabbit, provided its relative activity is evaluated by comparison to a suitable reference material.

With the advent of crystalline *d*-tubocurarine chloride, the unit has been expressed necessarily (see footnote 4) in terms of weight of a particular lot of this alkaloid, i.e., one unit = 0.155 mgm. of the pentahydrate form. Since its potency in humans has been adequately established, some clinicians have reported its dosage in mgm. of the alkaloid instead of units, but actually administered it in units. Unfortunately, this had led to confusion of the number of units of activity and the number of mgm. of this drug injected in at least two clinical reports (33, 34), and has occasioned inquiries as to the safety of the suggested high doses which resulted from the erroneous dosage statement. Needless to say, this serious error was immediately corrected in both instances (35, 36).

SUMMARY

1. The head-drop assay in the rabbit has been employed for the biological determination of potency of curares having an onset and duration of action comparable to that of *d*-tubocurarine chloride. Qualitatively similar crude or purified curare preparations standardized by this method show uniform activity in clinical use, although the relative activity of various curare compounds in the rabbit and man may be different.

2. The endpoint of the test, head-drop, is relaxation of the neck muscles to such a degree that the animal's head cannot be raised or turned in response to a physical stimulus.

3. In the assay the reference standard and test sample of curare are each injected intravenously into at least eight rabbits in a crossover pattern on two consecutive days at the rate of 0.1 cc. of a 2 unit per cc. solution every fifteen seconds without regard to body weight. Each animal must have received within seven days prior to use in the test at least two such titrations to head-drop. For routine assays the solution of test sample to be injected should have a potency within ± 10 per cent of the 2 units per cc. reference solution prepared from a standard sample of *d*-tubocurarine chloride pentahydrate of which 0.155 mgm. equals 1 unit of curare activity.

4. The relative potency of the diluted sample is calculated as the geometric mean of the ratios of the volume of standard to that of sample necessary to produce head-drop in each animal. The expected standard error of the geometric mean of the individual ratios, when sixteen rabbits are used in a crossover design, is 1.1 per cent.

5. The effect of rate of injection on head-drop dosage, recovery time and sharpness of the endpoint have been analyzed, and the limitations of the head-drop method and of other procedures studied have been discussed.

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REFERENCES

1. BENNETT, A. E.: *Am. J. Psychiat.*, **97**: 1010, 1941.
2. HOLADAY, H. A.: U. S. Patents #2,397,417 & #2,401,278.
3. DUTCHER, J. D.: *J. Am. Chem. Soc.*, **68**: 419, 1946.
4. MÖLLER, K. O.: *Acta pharmacol. et toxicol.*, **2**: 393, 1946.
5. SCHMIDT, J. L., AND CHASE, H. F.: *Proc. Soc. Exper. Biol. & Med.*, **65**: 13, 1947.
6. CHASE, H. F., SCHMIDT, J. L., AND BHATTACHARYA, B. K.: *THIS JOURNAL*, **91**: 236, 1947.
7. BURKE, J. C., AND LINEGAR, C. R.: *Fed. Proc.*, **7**: 208, 1948.
8. CHASE, H. F., LEHMAN, A. J., AND RICKARDS, E. E.: *THIS JOURNAL*, **82**: 266, 1944.
9. MARSH, D. F., AND PELLETIER, M. H.: *THIS JOURNAL*, **92**: 127, 1948.
10. EVERETT, G. M.: *THIS JOURNAL*, **92**: 236, 1948.
11. MARSH, D. F., AND PELLETIER, M. H.: *THIS JOURNAL*, **92**: 454, 1948.
12. KIMURA, K. K., AND UNNA, K. R.: *Fed. Proc.*, **7**: 233, 1948.
13. MARSH, D. F.: *Fed. Proc.*, **6**: 354, 1947.
14. MARSH, D. F., SLEETH, C. K., AND TUCKER, E. B.: *THIS JOURNAL*, **93**: 109, 1948.
15. PELLETIER, M. H., AND MARSH, D. F.: *Fed. Proc.*, **7**: 248, 1948.
16. MARSH, D. F., AND HERRING, D. A.: *Fed. Proc.*, **8**: 318, 1949.
17. SKINNER, H. G., AND YOUNG, D. M.: *THIS JOURNAL*, **91**: 144, 1947.
18. UNNA, K. R., AND KIMURA, K. K.: *Fed. Proc.*, **6**: 378, 1947.
19. VAN MAANEN, E. F.: *Fed. Proc.*, **7**: 261, 1948.
20. ALLMARK, M. G., AND BACHINSKI, W. M.: *J. Am. Pharm. A. (Scient. Ed.)*, **38**: 43, 1949.
21. COLLIER, H. O. J., PARIS, S. K., AND WOOLF, L. I.: *Nature*, **161**: 817, 1948.
22. CHOU, T. C.: *Brit. J. Pharmacol.*, **2**: 1, 1947.
23. BULBRING, E., AND CHOU, T. C.: *Brit. J. Pharmacol.*, **2**: 8, 1947.
24. GARCIA DE JALON, P. D., AND WEST, G. B.: *Nature*, **169**: 841, 1947.
25. KENZLER, C. J.: *Fed. Proc.*, **8**: 307, 1948.
26. TORDA, C., AND WOLFF, H. G.: *Am. J. Physiol.*, **147**: 384, 1946.
27. GARCIA DE JALON, P. D.: *Quart. J. Pharm. & Pharmacol.*, **20**: 28, 1947.
28. PICK, E. P., AND RICHARDS, G. V.: *Proc. Soc. Exper. Biol. & Med.*, **67**: 329, 1948; *Arch. internat. de pharmacodyn. et de therap.*, **76**: 183, 1948.
29. COHNBERG, R. E.: *J. Lab. & Clin. Med.*, **31**: 866, 1946.
30. KING, H.: *J. Chem. Soc. (London)*, **1381**, 1935.
31. WINTERSTEINER, O., AND DUTCHER, J. D.: *Science*, **97**: 467, 1943.
32. WEST, R.: *J. Physiol.*, **91**: 437, 1938.
33. KUHN, R. A., AND BICKERS, D. S.: *New England J. Med.*, **238**: 615, 1948.
34. WEED, M. R., PURVIS, D. F., AND WARNE, R. D.: *J.A.M.A.*, **138**: 1087, 1948.
35. Correspondence: *New England J. Med.*, **238**: 922, 1948.
36. *J.A.M.A.*, **139**: 45, 1949; **139**: 397, 1949.

METABOLIC TRANSFORMATIONS OF TRICHLOROETHYLENE¹

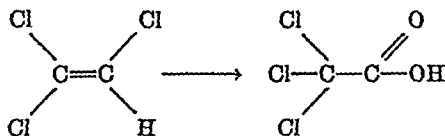
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The isolation of trichloroacetic acid from urine of dogs that had inhaled trichloroethylene vapor was reported by Barrett and Johnston (1) in 1939, and studies of the excretion of this metabolic product were continued by Barrett, Cunningham, and Johnston (2). Powell (3) also was able to isolate trichloroacetic acid from urine of human patients anesthetized with trichloroethylene. Powell's measurements of the concentration of trichloroacetic acid in plasma and urine indicated that the formation of the acid may continue for more than two days after a single period of anesthesia. The largest amount of trichloroacetic acid produced in any one of these patients appears to be somewhat more than 4 grams.

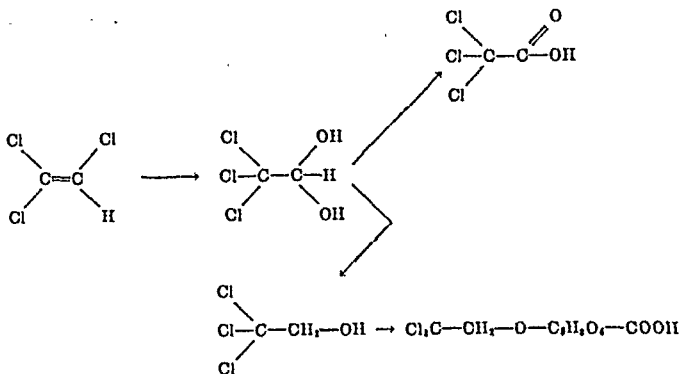
The transformation of trichloroethylene to trichloroacetic acid involves oxidation, saturation of the carbon-carbon double bond, and migration of a chlorine atom from one carbon atom to the other.



To trace the course of the processes that result in these changes is a problem of some complexity. Consideration first of the oxidative aspect of the reaction suggests that in the course of the conversion of trichloroethylene to trichloroacetic acid a structurally stable compound in an intermediate state of oxidation may be formed. Such a compound that might be presumed likely to occur as an intermediate is chloral hydrate, since chloral hydrate is known to be oxidized in part to trichloroacetic acid by the dog (4). However, chloral hydrate is also reduced *in vivo* to trichloroethanol; and of a dose of chloral hydrate injected in a dog, much the greater quantity is converted to the alcohol (4). Consequently, if chloral hydrate is assumed to be an intermediate product in the reaction, it must be expected that trichloroethanol will be produced as well as trichloroacetic acid.

Hitherto trichloroethanol had not been searched for as a metabolic product of trichloroethylene. The glucuronide, the principal form in which trichloroethanol is excreted, does not give the Fujiwara color reaction, nor is it extractable with ether. Evidence of its presence accordingly would not have appeared in the experiments cited above.

¹ Presented in part at the Thirty-ninth Annual Meeting of the American Society for Pharmacology and Experimental Therapeutics, April 22, 1949 (Fed. Proc., 8: 278, 1949).



In order to explain the slow production of trichloroacetic acid, it would have to be assumed that the conversion of trichloroethylene to chloral hydrate is very slow. Reactions that would occur subsequently (*viz.* oxidation of chloral hydrate to trichloroacetic acid, reduction of chloral hydrate to trichloroethanol, conjugation of trichloroethanol with glucuronic acid) being relatively rapid, neither chloral hydrate nor trichloroethanol could be expected to accumulate in any high concentrations in the body; but the urine would be expected to contain considerable amounts of the glucuronide of trichloroethanol, "urochloralic acid".

It was the hypothesis of chloral hydrate being a metabolic product of trichloroethylene that suggested the experiments reported here. As will be shown, the results have proved to be in general accord with the predictions outlined above.

MATERIALS. Trichloroethylene was purchased from The Matheson Co., Inc. Freedom from contamination with trichloroethanol or chloral hydrate in amounts sufficient to be of significance in this investigation was demonstrated in the following manner. A solution of 1 gm. of trichloroethylene in 100 cc. of heptane was shaken with $\frac{1}{4}$ its volume of water. Analysis of the aqueous phase for trichloroethanol and chloral hydrate by methods described in an earlier paper (4) failed to reveal the presence of either substance. By addition of known amounts it was shown that as little as 2 mgm. of trichloroethanol or 0.2 mgm. of chloral hydrate per gm. of trichloroethylene would be detectable by this procedure.

ADMINISTRATION OF TRICHLOROETHYLENE TO DOGS. A few minutes before the period of inhalation, an intramuscular dose of 0.1 mgm. per kgm. of atropine sulfate was given to reduce salivary and mucous secretion. Air bubbled through trichloroethylene was delivered through a loosely fitting mask. The total period of inhalation was 1 hr. Anesthesia was induced rapidly and maintained at an inconstant but rather deep level. Toward the end of the period the depth of anesthesia was increased almost to the point of respiratory arrest.

ANALYTICAL METHODS. *Conjugated trichloroethanol in urine.* Urine was acidified to about pH 2 with sulfuric acid. An additional 0.5 cc. of concentrated sulfuric acid was then added to each 10 cc. of urine. A 1 cc. sample of the acidified urine was sealed in a glass tube and heated in a bath of boiling water for 10 hr. The tube was then opened and the contents

washed out with water. After appropriate dilution (usually 1:10), trichloroethanol was determined by the method previously described for plasma (4).

Trichloroacetic acid and chloral hydrate in plasma. The methods of an earlier study (4) were used.

Trichloroacetic acid in urine. The method was essentially the same as that used for plasma.

Trichloroethanol in plasma. Modifications of the method used previously (4) were introduced to increase the sensitivity. The size of the plasma sample was increased. The heptane extract was extracted with two portions of water, and the salted aqueous extract was extracted with three portions of pentane. These modifications made possible the determination of concentrations of trichloroethanol as low as 1 mgm. per l. of plasma.

CONJUGATED TRICHLOROETHANOL IN URINE. *Identification of trichloroethanol.* When urine from dogs that have inhaled trichloroethylene is acidified and heated, a substance is formed that is determinable as trichloroethanol by the colorimetric method (4). The identification of this substance as trichloroethanol was established by the following experiments. Urine from a dog exposed to trichloroethylene was acidified as described under "Methods" and heated on a steam bath under a reflux condenser for 10 hr. By the procedures described in an earlier paper (4), it was found that the colorimetrically determined "trichloroethanol" in this urine had a distribution coefficient between heptane and water of 0.43 (for authentic trichloroethanol: 0.41) and that oxidation was necessary for the production of color in the Fujiwara reaction. A sample of 100 cc. of the acidified and heated urine, estimated colorimetrically to contain 197 mgm. of trichloroethanol, was neutralized with sodium hydroxide and subjected to steam distillation. The analytical method indicated that the first 50 cc. of distillate contained 166 mgm. of trichloroethanol (84 per cent of the total). This was saturated with sodium chloride and extracted with pentane in a manner calculated to remove 98 per cent of the alcohol. The pentane was removed by the successive evaporation of 10 cc. portions added to 2 cc. of pyridine. To the pyridine residue were added 250 mgm. of *p*-nitrobenzoyl chloride. From the reaction mixture were isolated 230 mgm. of crystals melting at 68.0–68.2° C. Trichloroethyl-*p*-nitrobenzoate prepared from synthetic alcohol melted at 68.0–68.4° C.² A mixture of approximately equal parts of the product from urine with the synthetic melted at 68.0–68.4° C. The yield of *p*-nitrobenzoate corresponds to 71 per cent of the trichloroethanol estimated to be in the pentane extract and 58 per cent of the total trichloroethanol estimated to be in the original urine. It may be concluded that the material determined colorimetrically in urine as "trichloroethanol" actually consists practically in its entirety of trichloroethanol. Presumably it all arises from the hydrolysis of the glucuronide, a compound known to be present (*v. i.*). This is the only conjugated form of trichloroethanol that has been described, and from what is known of analogous compounds no other type of conjugation would be expected.

² The melting point of this compound has previously been reported (5, 6) as 71° C. The ester studied here was prepared both in pyridine and by the Schotten-Baumann procedure, and the melting point reported above (corrected) was not changed by repeated crystallizations from ethanol and from heptane. The compound contained 35.8 per cent chlorine (theoretical for $C_9H_8O_4N Cl$: 35.6 per cent).

Isolation of trichloroethylglucuronide from urine. Urine from dogs that had inhaled trichloroethylene was subjected to the procedure outlined by Külz (7) for the isolation of the sodium salt of urochloralic acid. In this way there was obtained a white crystalline substance containing 30.3 per cent chlorine (theoretical for $C_2H_3O_7Cl_2Na$: 30.6 per cent). For an aqueous solution of 12 gm. per l., $[\alpha]_D^{21} = -59^\circ$.³ When 2.5 mgm. of this material was dissolved in 1 cc. of 2*N* hydrochloric acid and the solution heated in a sealed tube at 100° C. for 9 hr., the colorimetric procedure showed that 0.92 mgm. of trichloroethanol had been produced (86 per cent of the theoretical). No conditions of hydrolysis were found that resulted in a higher yield of alcohol. The distribution coefficient of the colorimetrically determined "trichloroethanol" between heptane and water was 0.35, and no color was produced without oxidation. The solution that had been subjected to acid hydrolysis gave the naphthoresorcinol test for glucuronic acid. This material isolated from urine can then be considered to consist,

TABLE 1

Excretion of trichloroethanol and total production of trichloroacetic acid following anesthesia with trichloroethylene

DOG	WEIGHT	TRICHLOROETHANOL EXCRETED*	TRICHLOROACETIC ACID PRODUCED**
	kgm.	grams	grams
1	14.0	1.52	0.70
2	8.2	0.53	0.16

* Nearly all conjugated.

** Estimated from plasma concentrations and urinary excretion.

in very large part at least, of the glucuronide of trichloroethanol, the same substance found by earlier workers as a metabolic product of chloral hydrate and of trichloroethanol.

Quantitative studies of excretion. Measurements have been made of the amount of trichloroethanol liberated in urine by acid hydrolysis. Although the conditions of hydrolysis probably do not permit strictly quantitative recovery of trichloroethanol,⁴ the analytical results are obviously significant as minimal values and probably represent a high proportion of the conjugated alcohol present. If the values are considered to have only this significance, the conclusions reached in this paper are satisfactorily supported.

In table 1 are shown the total amounts of trichloroethanol found in the urine of two dogs that had inhaled trichloroethylene vapor for 1 hr. Nearly all of this trichloroethanol is conjugated. The amount of free trichloroethanol found in the

³ The rotations of sodium urochloralate published by Külz (7) were measured in white light with a saccharimeter with quartz-wedge compensator. If the rotatory dispersion is assumed to be the same as that of sucrose, $[\alpha]_D^{20} = -65^\circ$. This value is not strictly comparable with that reported above.

⁴ Presumably pure sodium urochloralate was added to a sample of normal urine in a concentration of 2.5 gm. per l. and the urine subjected to the procedure described under "Methods". The amount of trichloroethanol found was 70 per cent of the theoretical.

urine of Dog 1 was only 0.6 per cent of the total and in that of Dog 2, 1.6 per cent of the total.

Before an estimate could be made of the amount of trichloroethanol actually produced in these dogs, it would be necessary to know the proportion of trichloroethanol excreted. At later times the excretion of trichloroethanol in the same animals was measured following injection of the alcohol itself. In an effort to simulate the conditions of slow, sustained production from trichloroethylene, trichloroethanol was injected intramuscularly in several small doses over a period of 24 hr. The results are shown in table 2. Because quantitative recovery of trichloroethanol is probably not attained, the actual amounts excreted are likely to be somewhat higher than those found.⁶ However, since the same technique was used in both studies, the data of table 2 are comparable with those of table 1. It may be estimated then that the amounts of trichloroethanol actually produced from trichloroethylene are of the order of twice the amounts found in the urine as shown in table 1.

TABLE 2

Excretion of trichloroethanol (nearly all conjugated) following intramuscular injection of trichloroethanol in several doses over a period of 24 hr.

DOG	TOTAL DOSE OF TRICHLOROETHANOL	TRICHLOROETHANOL EXCRETED	
		Amount	Per Cent
	<i>grams</i>	<i>grams</i>	
1	3.75	1.57	42
1	2.0	1.10	55
2	1.0	0.66	66

The rate at which trichloroethanol is produced from trichloroethylene is also of interest. For the same experiments of those of table 1, average rates of excretion of conjugated trichloroethanol during the periods of urine collection are shown in fig. 1. The long continuation of the excretion is notable. Whether this is attributable to slow production or to slow excretion cannot be judged without a knowledge of the rate at which small amounts of trichloroethanol leave the body as the conjugate. The following experiment served to supply this information. A dog was given an intravenous dose of 5 mgm. per kgm. of trichloroethanol, a dose designed to give a blood concentration in the same range as those found after inhalation of trichloroethylene. Within the first 7 hr. after the injection, 65 per cent of the administered dose had appeared in the urine. Of this amount, 88 per cent had been excreted in the first 3 hr. The maximum rate of excretion, 23 mgm. per hr., was attained about 1 hr. after the injection. The processes of conjugation and excretion of trichloroethanol are thus shown to be relatively

⁶ Earlier estimates of the excretion of urochloralic acid (7-10) have been based on measurement of optical rotation of urine or on measurement of glucuronic acid. The proportions of chloral hydrate or trichloroethanol that have been reported to be excreted as urochloralic acid by dogs and rabbits are for the most part in the range of 40 to 60 per cent.

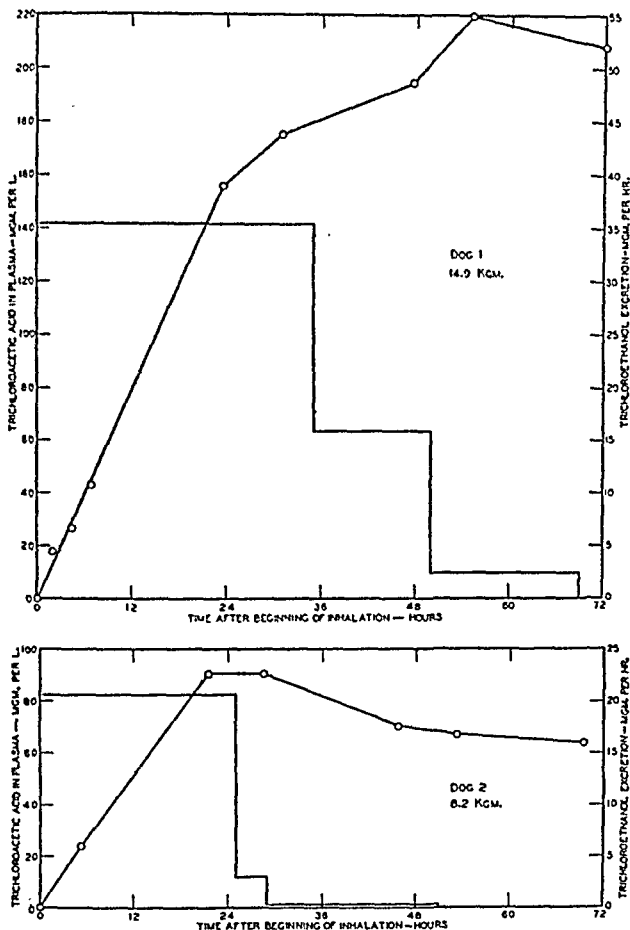


FIG. 1. Concentration of trichloroacetic acid in plasma and rate of excretion of trichloroethanol (nearly all conjugated) following a single exposure to trichloroethylene lasting 1 hr. Trichloroacetic acid concentrations are indicated by the circles. The average rate of excretion of trichloroethanol during a period of urine collection is indicated by a horizontal line.

rapid. The long sustained excretion after administration of trichloroethylene can only result from a slow, continuous production of trichloroethanol.

METABOLIC PRODUCTS IN PLASMA. Chloral hydrate was not found in the plasma of the dogs that had inhaled trichloroethylene. The method used could have detected concentrations as low as 8 mgm. per l. Free trichloroethanol was found in the plasma, but in low concentrations. In both dogs the highest concentration observed was 3 mgm. per l. This was found at 24 hr. in Dog 1 and at 5 hr. in Dog. 2. The low concentration of trichloroethanol in plasma is further evidence of the slowness of its production.

As shown in fig. 1, the concentration of trichloroacetic acid in the plasma rises slowly to reach quite high values. The maximum concentration is reached only after more than two days in Dog 1 and after about one day in Dog 2. This pattern is similar to that found by Powell (3) in human subjects. Trichloroacetic acid being very slowly eliminated, the plasma concentration of itself furnishes a rather good indication of the rate of production. Since little if any trichloroacetic acid is destroyed (11), an estimate of the total production may be made from the plasma concentration and the urinary excretion. The assumption was made that trichloroacetic acid is distributed in 20 per cent of the body weight. At some time after the plasma concentration had begun to decline, the total amount in the body was calculated in this way. This value was added to the amount excreted in the urine up to that time to give an estimate of the total amount of trichloroacetic acid produced. These estimates for the two dogs are included in table 1.

DISCUSSION. The experimental results seem consistent with the consequences of the hypothesis that trichloroethylene is initially converted to chloral hydrate. As predicted, trichloroethanol is produced, and indeed in larger amounts than trichloroacetic acid. If the amount of trichloroethanol actually produced is assumed to be about double the amount found in the urine, the ratio of alcohol production to acid production is still considerably lower than that found when a large dose of chloral hydrate is injected into a peripheral vein of a dog (4). This is not, however, necessarily indicative that both substances have not been derived entirely from chloral hydrate. If chloral hydrate were formed only at a site, such as the liver, where it is capable of oxidation to trichloroacetic acid (12), the proportion converted to acid would be larger than if chloral hydrate were introduced into the general circulation.

The rates at which trichloroethanol and trichloroacetic acid are formed may be considered as additional evidence of their origin. The simplest hypothesis that could explain the very slow formation of both the alcohol and the acid is that there is a single slow process yielding a substance that is their common precursor. Since the alcohol cannot be converted to the acid, nor the acid to the alcohol, the alternative hypothesis is that trichloroethylene undergoes two independent reactions or systems of reactions, both slow, leading to the formation of trichloroethanol and trichloroacetic acid, respectively. If chloral hydrate is the precursor of either product, it must also be the precursor of at least a part of the other. The most plausible hypothesis would appear to be that chloral hydrate is the precursor of all of both products.

Although the exact mechanism of the metabolic rearrangement has not been elucidated, it seems probable that the initiating process is the removal of one or two electrons from the trichloroethylene molecule, and that it is the deficiency of electrons that makes possible the migration of an electron pair. The first structurally stable product is probably chloral hydrate, since no over-all oxidation has occurred in the conversion of trichloroethylene to trichloroethanol, it seems unlikely that the rearrangement could take place through a non-oxidative route.

The long continuation of the appearance of metabolic products following a single exposure to trichloroethylene is remarkable. Evidently, considerable amounts of trichloroethylene are retained in some form in the body, and since any obvious pharmacological effects of the drug have disappeared, the physiological solution in fat may be sufficient to account for most of the retention. Trichloroethylene has been detected chemically by several workers at long intervals after exposure. Powell (3) found detectable amounts in blood and exhaled air of human patients 24 hr. after anesthesia. Barrett, Cunningham, and Johnston (2) detected the substance in fat, muscle, and heart of a dog killed 24 hr. after exposure. Of these tissues, fat contained much the highest concentration. Goss (13) found traces in tissues of guinea pigs killed 16 hr. after exposure.

From analyses of tissues, Barrett, Cunningham, and Johnston estimated that a dog anesthetized by an exposure to trichloroethylene vapor for 22 to 28 min. retains about 87 mgm. per kgm. immediately after the exposure. In the present study, Dog 1 converted 127 mgm. per kgm. and Dog 2 converted 73 mgm. per kgm. of trichloroethylene to recognizable metabolic products. Of the trichloroethylene present in the body at the end of a period of anesthesia, the proportion undergoing metabolic changes has not been determined, but it must be rather large.

The metabolism of trichloroethylene may then be pictured as follows. The initial reaction is oxidation accompanied by rearrangement, leading to the formation of chloral hydrate. This reaction proceeds at a very slow rate, a rate nearly independent of the concentration of trichloroethylene, until the stores of the substance in the body have been exhausted. The chloral hydrate so produced is converted by relatively rapid reactions both to trichloroethanol and to trichloroacetic acid. None of these products reach concentrations high enough to have any obvious pharmacological effects.

SUMMARY

After administration of trichloroethylene to dogs, trichloroethanol has been found in small amounts in the plasma and conjugated trichloroethanol has been found in large amounts in the urine. The glucuronide of trichloroethanol has been isolated from the urine. Trichloroethanol is produced in larger amounts from trichloroethylene by the dog than is trichloroacetic acid, a metabolic product previously identified.

The course of the metabolic transformations of trichloroethylene is discussed. It is suggested that trichloroethylene is initially converted to chloral hydrate,

which is the immediate precursor both of trichloroethanol and of trichloroacetic acid.

REFERENCES

1. BARRETT, H. M., AND JOHNSTON, J. H.: *J. Biol. Chem.*, **127**: 765, 1939.
2. BARRETT, H. M., CUNNINGHAM, J. G., AND JOHNSTON, J. H.: *J. Indust. Hyg. & Toxicol.*, **21**: 479, 1939.
3. POWELL, J. F.: *Brit. J. Indust. Med.*, **2**: 142, 1945.
4. BUTLER, T. C.: *THIS JOURNAL*, **92**: 49, 1948.
5. WILLSTÄTTER, R., AND DUISBERG, W.: *Ber. d. deutsch. chem. Ges.*, **56**: 2283, 1923.
6. GILMAN, H., AND ABBOTT, R. K., JR.: *J. Org. Chem.*, **8**: 224, 1943.
7. KÜLZ, E.: *Arch. f. d. ges. Physiol.*, **28**: 506, 1882.
8. THIERFELDER, H.: *Ztschr. f. physiol. Chem.*, **10**: 163, 1886.
9. MAYER, P., AND NEUBERG, C.: *Ztschr. f. physiol. Chem.*, **29**: 256, 1900.
10. AKAMATSU, M., AND WASMUTH, F.: *Arch. f. exper. Path. u. Pharmacol.*, **99**: 108, 1923.
11. PAYKOÇ, Z. V., AND POWELL, J. F.: *THIS JOURNAL*, **85**: 289, 1945.
12. BUTLER, T. C.: *THIS JOURNAL*, **95**: 360, 1949.
13. GASQ, M.: *Étude toxicologique et hygienique des solvants chlorés acéthyléniques*. Delmas, Bordeaux, 1936. (From quotations by von Oettingen, W. F.: *J. Indust. Hyg. & Toxicol.*, **19**: 349, 1937.)

STREPTOMYCIN: CLEARANCE AND BINDING TO PROTEIN

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The relationship of the dose of streptomycin per kilogram of body weight and the rate of decrease of the concentration of the drug in the blood in man and dog has been reported in a previous paper (1). A simple logarithmic equation was found to describe the observed time-concentration relationship and the numerical values of the constants were calculated from the experimental data. No urinary excretion data were obtained in these experiments. It was realized, however, that the slope of the straight lines and the "apparent" volume of distribution were related in a simple fashion to the renal clearance under certain well defined conditions. Such a quantitative relation between the renal clearance of a substance and the exponential decrease of its concentration in the blood has been clearly discussed by Newman, Bordley and Winternitz (2). If the equation

$$(1) \ln P = k \cdot t + \ln P_0$$

describes the course of the drug concentration in the blood, then, if certain basic assumptions are made,

$$(2) k = \frac{C}{V_e}$$

where C is the value for the urinary clearance in cc. per minute, k the experimentally determined slope, and V_e , the volume of distribution expressed in cc.¹ The basic assumptions underlying equations (1) and (2) are: a) That the fluid compartment throughout which a given quantity, M , of the substance is distributed, is constant; b) That the substance is not destroyed in the body, but is excreted unaltered; and c) That the amount of M withdrawn from the fluid compartment, V_e , per unit time is solely excreted by the kidney. Then

$$(3) - \frac{dM}{dt} = U \cdot V = C \cdot P$$

where U and V have the usual meaning and dimensions employed in urinary clearance studies.

¹ Some of the symbols used in the previous paper (1) were changed in order to conform with the notation commonly used in the presentation of clearance data. The concentration of the drug in the plasma denoted as C in the previous paper is termed P in this discussion. Furthermore, the numerical value for the slope k as derived from equation (1), is expressed as a fraction per minute with logarithms to the base e ; while in the previous paper K was expressed as a fraction per hour with logarithms to the base 10.

Equation (2) relates the slope, k , to the urinary clearance, C , and a value for C can be obtained from plasma data alone, if the volume of distribution, V_c , is known. Newman, Bordley and Winternitz (2) in their study on the urinary clearance of mannitol have shown, assuming a value for V_c of 20 per cent of body weight, that there is excellent agreement between the values for the clearance obtained from measurements of the urinary excretion and those from plasma data alone. In cases in which it is known that an injected substance is not destroyed in the body and is excreted solely by the kidney, it is unnecessary to make an assumption as to the value of the volume of distribution. According to equation (1), extrapolation to zero time of the straight line obtained by plotting the logarithm of the observed concentrations, P , against time, leads to a value for P_0 ; the "apparent" volume of distribution, V_c , can then be obtained by dividing the total amount of the substance injected by P_0 . The value for the clearance, C , can thus be calculated from plasma values alone according to equation (2). Conversely, the absence of appreciable destruction of a substance in the body and its excretion solely by the kidney is indicated, if the values for the volume of distribution, V_c , and the renal clearance, C , calculated from the measurement of the urinary excretion, agree with the values calculated from the plasma data.

The urinary clearance of streptomycin has been determined by Adcock and Hettig (3) in man and dog using a microbiological assay procedure, and with more analytical accuracy by Marshall (4) using a chemical determination. The reported results indicate that the renal clearance of streptomycin is lower than the values assumed for the glomerular filtration rate. The work of H. Smith (5) emphasizes, however, the necessity of establishing the glomerular filtration rate simultaneously and independently in each animal used for clearance studies in order to reach significant conclusions concerning the renal excretion mechanism of a substance.

Considerable analytical difficulties are encountered if the normal glomerular filtration is to be measured by the usual test substances in the presence of streptomycin. Streptomycin interferes with the analytical methods for inulin, mannitol, and creatinine although these substances do not influence the fluorometric determination of streptomycin. The methods for the determination of inulin and mannitol are more or less unspecific carbohydrate reactions given to some extent by streptomycin, while the determinations of creatinine are based on the reaction of the guanido group which is also present in streptomycin. Newman, Gilman and Philips (6) introduced the use of thiosulfate ion for the measurement of the glomerular filtration rate in man and dog by demonstrating that the values obtained were identical with those for the inulin clearance. Since the presence of streptomycin and thiosulfate in blood or urine does not interfere with the assay procedure for either substance, the thiosulfate clearance was chosen as the measure of the glomerular filtration rate in our experiments.

EXPERIMENTAL. Female dogs, unanesthetized, trained to the procedure, and varying in weight from 13.5 to 15.0 kgm. were used in the experiments. Their surface area was calculated from the formula $A = W^{2/3} \times 11.2$ (7). All animals received 20 cc./kgm. of water on

the evening preceding the experiment and the same amount just prior to the test. In order to obtain wide variations in the rate of urine flow, a third dose of water was given 2.5 hours after the start of the experiment in three cases. Blood and urine samples were taken for blank determinations just prior to the injection of the drugs.

The urine was obtained by means of an indwelling catheter and the bladder was rinsed with 5 cc. of water at the end of each interval. The urine volumes and flow rates were corrected for the volume of water used in rinsing the bladder.

A recrystallized sample of streptomycin trihydrochloride calcium chloride double salt with a potency of 730 microgm./mgm. was used and the calculated quantity of the drug dissolved in 2 cc. of water was injected intramuscularly. Aliquots of the injection solutions were assayed by two chemical methods, the maltol (8) and the hydrazine methods (9). Thiosulfate was injected intravenously at the same time as the streptomycin in a dose of 200 mgm. $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ per kgm. of body weight. A freshly prepared 10 per cent solution was used and the thiosulfate content was checked by titration.

Blood and urine samples were obtained at approximately half-hour intervals, the exact time being noted in each case and used for the calculations. Streptomycin in the blood and urine was determined by the fluorometric method previously described (9). Thiosulfate in blood and urine was determined according to Newman, Gilman and Philips (6).

RESULTS. The results of a typical experiment obtained with a dose of 5,000 microgm. of streptomycin per kgm. is represented graphically in fig. 1.

The slopes of the hemilogarithmic plot of the P and U-V values for streptomycin and thiosulfate respectively are essentially equal, indicating that in the range of the experiment the clearance of each of the substances was independent of its plasma concentration. The ratio of the slopes of the lines for the streptomycin and thiosulfate plasma values does not indicate the ratio of the filtration rates of the two substances, since according to equation (2) the filtration rate also depends on the respective volume of distribution.

To express the graphical presentation of figure 1 numerically for comparison with four similar experiments performed with different doses of streptomycin, the slope k , and the intercept P_0 , of the straight lines of the plasma values extrapolated to zero time, were determined by the method of least squares. From the values for P_0 the "apparent" volumes of distribution were calculated for both streptomycin and thiosulfate, and were expressed in table I as per cent of body weight. From this volume of distribution and the corresponding k value the renal clearance was then calculated according to equation (2). All these figures are reported in table I under "Data obtained from plasma values".

The urinary clearances were also calculated in the customary manner from the U-V values and the corresponding P values according to equation (3) and the volume of distribution then derived by substituting this value in equation (2). These figures are reported in table I under "Data obtained from urinary values".

All clearances are recalculated to 1.73 m² surface area of the animal. Each value under streptomycin is the average of the measurement of six periods of about one-half hour, and each value for thiosulfate is the average of four corresponding one-half hour periods. Standard deviations have been recorded whenever the figures represent averages or were obtained by fitting the data to a straight line by the method of least squares.

Equation (2) describes the plasma clearance of a substance irrespective of

its mode of disappearance, while equation (3) measures only the clearance due to excretion into the urine. Therefore, if the values calculated from plasma data alone and from the conventional clearance formula are identical, it indicates that the substance disappears from the plasma only by urinary excretion. Streptomycin is an example of such a case since the values for the clearance and volume of distribution from both plasma and urinary data are essentially in

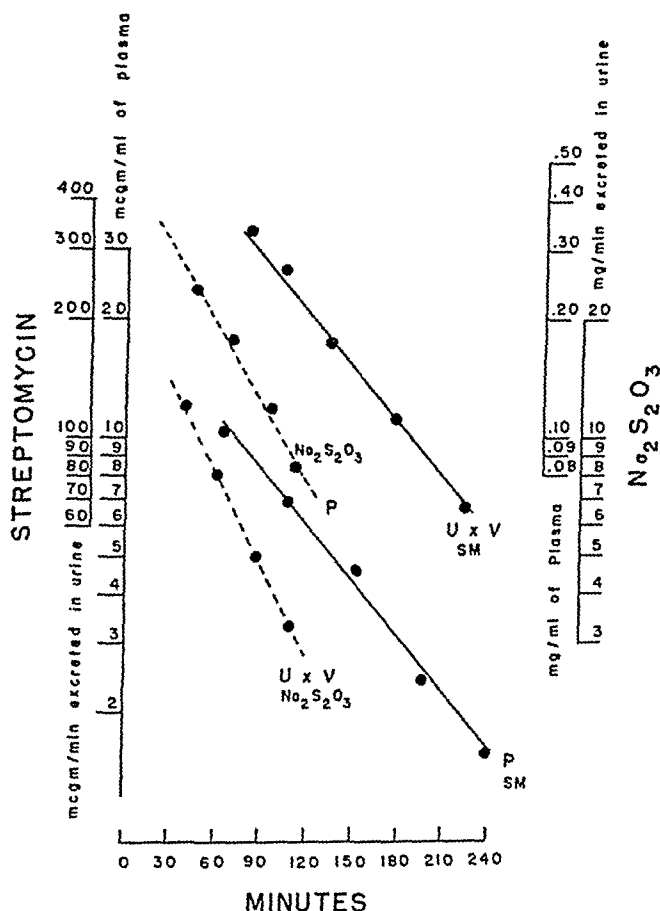


FIG. 1. Concentrations in plasma and rate of excretion in the urine of simultaneously administered streptomycin and thiosulfate ion.

agreement. Thiosulfate, on the other hand, is known to be partially destroyed in the body (6), and is a case in which the clearance values obtained from plasma data are higher than those obtained by the conventional urinary clearance formula.

The fact that essentially all of the injected streptomycin was excreted unchanged in these experiments on dogs was further corroborated by determining the total streptomycin excreted over the 24 hour period following the injection.

TABLE I
Clearance of Streptomycin from Plasma of Dogs

STREPTOMYCIN INJECTED	STREPTOMYCIN DATA OBTAINED FROM						N-14-DATA OBTAINED FROM						URINARY STREPTOMYCIN CLEARANCE AS % OF URINARY CLEARANCE
	Plasma values			Urine values			Plasma values			Urine values			
	k · 10 ³	Volume of Distribution as % of Body Weight	Clearance per 1.73 m ² per 1.73 m ³	Volume of Distribution as % of Body Wt. Av. of 6 periods	Clearance per 1.73 m ² Av. of 6 periods	k 10 ³	Volume of Distribution as % of Body Weight	Clearance per 1.73 m ² per 1.73 m ³	Volume of Distribution as % of Body Wt. Av. of 4 periods	Clearance per 1.73 m ² Average of 4 periods			
											%	cc./min.	
microgram / kgm.												%	
5,000	1.09 ± 0.03	23.0 ± 1.5	92.5	24.1 ± 1.2	96.6 ± 4.9	1.50 ± 0.01	26.9 ± 1.3	118.0	19.6 ± 1.8	107.7 ± 10.0	89.7		
10,000	0.86 ± 0.03	20.5 ± 0.9	64.8	23.8 ± 2.8	75.3 ± 8.6	1.47 ± 0.02	22.8 ± 0.3	123.0	18.4 ± 3.0	99.3 ± 16.3	75.8		
20,000	0.68 ± 0.01	23.2 ± 0.3	58.1	28.9 ± 2.7	72.5 ± 6.8	1.81 ± 0.01	21.6 ± 0.5	148.0	17.2 ± 2.2	117.5 ± 15.3	61.7		
20,000	0.95 ± 0.02	20.2 ± 0.8	72.7	21.8 ± 0.8	78.1 ± 2.9	1.67 ± 0.02	22.2 ± 0.4	137.0	16.8 ± 1.3	101.0 ± 7.8	75.3		
40,000	0.69 ± 0.03	27.7 ± 1.1	71.1	30.0 ± 1.4	77.0 ± 3.5	1.59 ± 0.01	27.5 ± 0.8	163.0	20.7 ± 1.1	122.0 ± 6.5	63.0		

The recovery of streptomycin in three experiments was 99, 97 and 92 per cent respectively.

The volume of distribution of streptomycin is distinctly larger than the true value for thiosulfate and in accordance with the data previously reported (1) is somewhat higher than the assumed volume of total extracellular fluid of about 20 per cent.

Comparison of the clearance values for streptomycin and thiosulfate obtained from urinary data leads to the conclusion that the urinary clearance of streptomycin is in all cases below the simultaneously measured glomerular filtration

TABLE 2
Clearance of Streptomycin from Plasma of Dogs Calculated from Plasma Data

STREPTOMYCIN INJECTED	SURFACE AREA	K · 10 ²	VOLUME OF DIS- TRIBUTION	CLEARANCE	CLEARANCE PER 1.73 m ²
microgm./kgm.	m ²	min ⁻¹	cc.	cc./min.	cc./min.
2,500	.582	1.07	2150	22.9	67.9
2,500	.582	1.19	1870	22.2	66.0
5,000	.577	1.26	1890	23.8	71.3
5,000	.633	1.09	3100	33.8	92.5
5,000	.435	1.14	1430	16.3	64.8
10,000	.584	1.00	2350	23.5	69.4
10,000	.643	0.83	3500	29.2	78.5
10,700	.635	0.86	2770	23.8	64.8
20,000	.580	0.84	3050	25.5	76.0
20,000	.562	0.71	2750	19.7	60.5
20,000	.681	0.95	3030	28.6	72.7
20,000	.641	0.68	3200	21.6	58.4
20,500	.613	0.69	3010	21.5	60.6
20,500	.624	0.69	3240	22.4	61.9
40,000	.598	0.83	3230	26.7	77.3
40,000	.603	0.72	3250	23.5	67.4
40,000	.641	0.69	3810	26.4	71.1
				Average 69.5	
				Std. Dev. ±8.4	

rate. The data in table 1 indicate that the filtration rate is not influenced by the per kgm. dose injected within the limits tested. The urinary clearance of streptomycin is on the average 70 per cent of the corresponding glomerular filtration rate.

Since the preceding data and discussion indicate that valid figures for the clearance of streptomycin can be obtained from plasma values alone, it was of interest to calculate for comparison the plasma clearance for man and dog from data reported in the previous paper of this series (1). The values for k and the "apparent" volume of distribution were substituted in equation (2). The data for the dog are reported in table 2 and include the five experiments recorded in table 1 of this paper and the twelve experiments in table 1 of the previous paper (1). The data in table 3 were all calculated from the experiments on man reported

in table 2 of the previous paper. It is apparent that the clearances per 1.73 m² body surface are essentially the same in man and dog. It was previously pointed out that the values for the volume of distribution and K were quite constant within the dosage range from 4,000 to 20,000 microgm./kgm. in man and 10,000 to 40,000 microgm./kgm. in dogs. At lower levels, however, the results were irregular and in general a small volume of distribution coincided with a high value for K. Such a behavior would be expected, if the clearance is essentially constant and equation (2) applies. In tables 2 and 3 all the results including the very low doses were summarized. This is justified, since individual figures

TABLE 3

Clearance of Streptomycin from Plasma of Man Calculated from Plasma Data

STREPTOMYCIN INJECTED	SURFACE AREA	K·10 ³	VOLUME OF DIS- TRIBUTION	CLEARANCE	CLEARANCE PER 1.73 m ²
microgm./kgm.	m ²	min ⁻¹	m ³	cc./min.	cc./min.
1,600	1.84	1.39	7,100	98.5	92.6
1,700	1.66	0.26	17,900	47.0	49.3
2,100	1.61	0.93	8,050	76.5	81.6
2,500	2.04	0.27	20,600	55.0	46.5
2,600	1.95	0.48	17,700	85.5	75.9
2,900	1.69	0.57	11,400	65.0	68.4
3,800	1.59	0.44	15,350	67.0	72.9
4,000	2.02	0.45	22,400	100.0	85.7
4,200	1.75	0.39	18,100	70.3	69.5
6,900	1.66	0.52	14,500	75.0	78.0
8,200	1.85	0.48	17,400	83.9	78.3
11,600	1.28	0.39	10,200	39.9	54.0
13,900	1.77	0.48	13,850	66.0	64.4
14,200	1.72	0.45	16,600	78.7	79.2
14,200	1.69	0.48	15,100	73.0	78.8
20,100	1.77	0.37	16,100	59.5	58.3
20,200	1.87	0.25	22,300	54.8	50.7
				Average 69.5	
				Std.Dev. ±13.7	

indicate that the clearances were essentially constant and equation (2) applicable, irrespective of the "regular" or "irregular" values for K and the volume of distribution.

In interpreting the experimental result that the clearance of streptomycin was significantly lower than the simultaneously observed filtration rate, the possibility of tubular reabsorption must be considered. If tubular reabsorption occurs, the filtration rate should be dependent on variations either in the plasma concentration of the drug or in the rate of urine flow(5). In figures 2 and 3 the essential independence of the clearance of streptomycin from either plasma concentration or urine flow is clearly demonstrated. Since single injections of the drug were used, the plasma concentrations in every case varied over a considerable range. In each experiment, furthermore, the urine flow varied at least four fold, and in three experiments nearly tenfold.

Binding of Streptomycin to Plasma Proteins. Since tubular reabsorption of streptomycin has been virtually excluded, the low filtration rate must be due to a mechanism which makes a part of the streptomycin unavailable for diffusion through the glomerular membrane. Protein binding is a plausible mechanism and

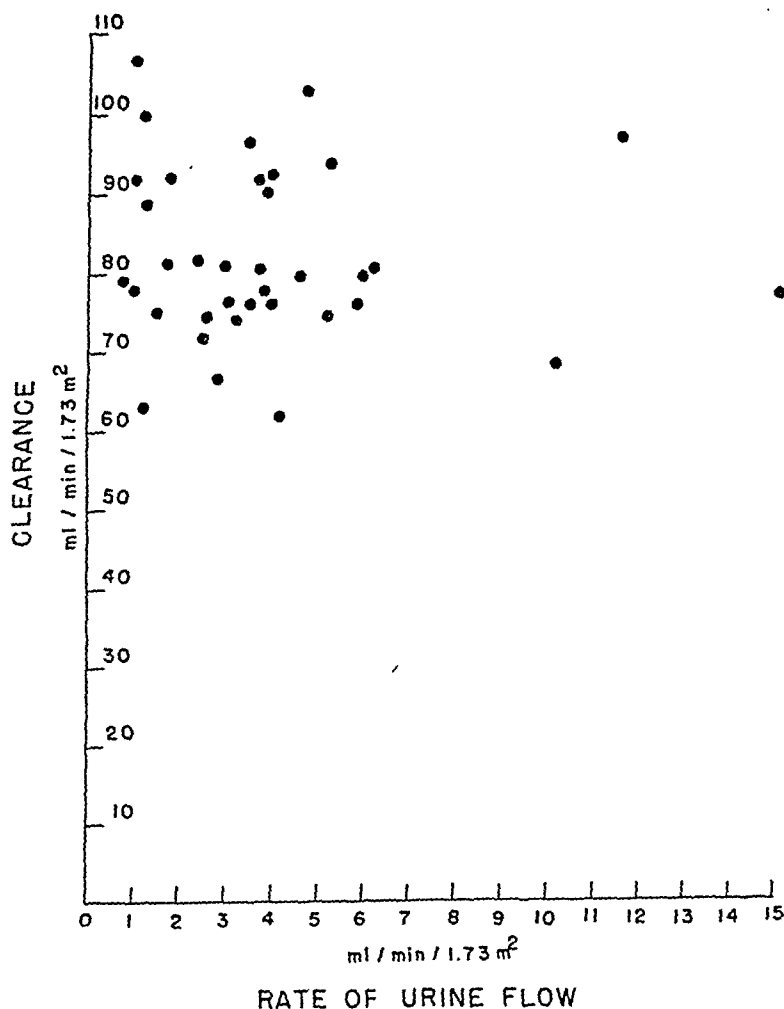


FIG. 2. Relationship of urinary clearance of streptomycin to the rate of urine flow

consequently the binding of streptomycin to plasma proteins was studied by dialysis and ultrafiltration experiments.

EXPERIMENTAL. We are indebted for the techniques of both the diffusion and ultrafiltration experiments to C. S. Smith (10). In the dialysis experiments 3 cc. of plasma contained in a cellophane bag (#133 of the Viscose Corporation) tightly closed with surgical thread was immersed in 15 cc. of Ringer solution containing varying amounts of streptomycin. The Ringer solution and the immersed bag were shaken for various lengths of time in a

closed vessel on a mechanical shaker at room temperature. Streptomycin was determined in the fluids inside and outside of the bag at the times indicated in table 4.

For the ultrafiltration experiments loops of cellophane tubing filled with 5 cc. of plasma containing streptomycin were suspended by means of a rubber stopper in 15 cc. centrifuge tubes. Centrifugation for ten minutes at about 1500 r.p.m. yielded approximately 0.2 cc.

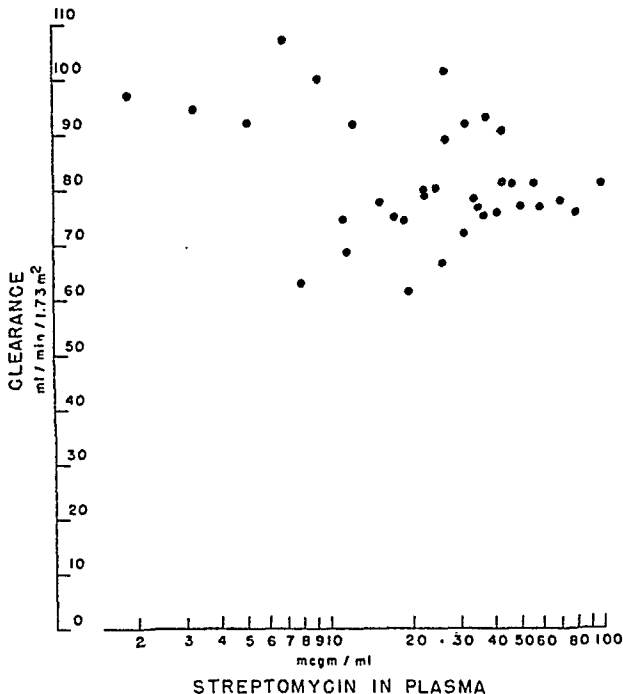


FIG. 3. Relationship of urinary clearance of streptomycin to the concentration in the plasma.

ultrafiltrate. The first filtrate was discarded, since it was diluted with water contained in the membrane. The subsequent filtrates were found to be at equilibrium and 0.2 cc. was used for each fluorometric analysis. Not more than three cuts or a total of 0.6 cc. of ultrafiltrate were removed in order to avoid appreciable changes in the protein concentration inside the membrane.

RESULTS. Tables 4 and 5 clearly demonstrate that protein binding of streptomycin does occur. In the dialysis experiment equilibrium was reached between

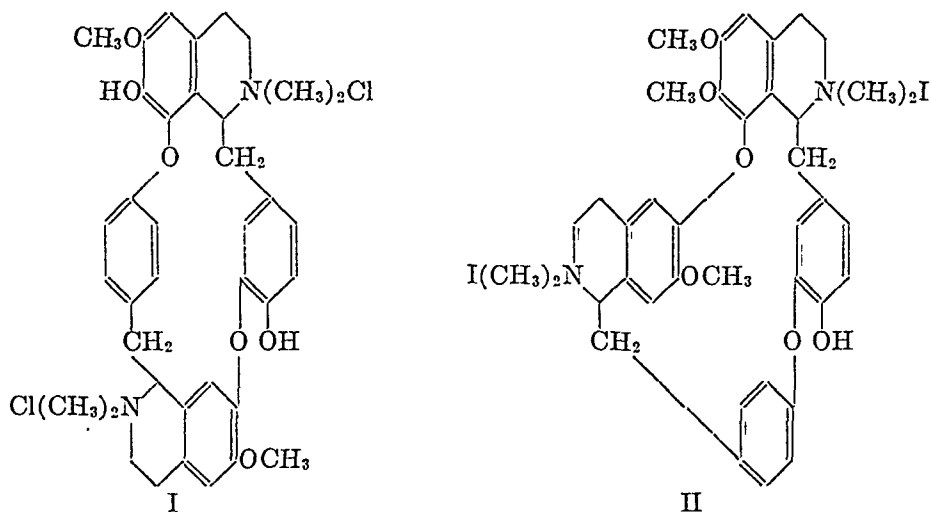
THE CURARIFORM ACTIVITY OF N-METHYLOXYACANTHINE

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Oxyacanthine¹ was isolated in pure form from *Berberis vulgaris* by Hesse (1) and its structure elucidated by von Bruchhausen and Schultze (2). Examination of its formula reveals that this tertiary alkaloid has many structural components found in d-tubocurarine. We have converted oxyacanthine into its quaternary derivative, N-methyloxyacanthine iodide and have compared its pharmacological activity with that of d-tubocurarine chloride.²



I. d-Tubocurarine (3): Molecular weight 624.7. Used as the dichloride. Pentahydrate, molecular weight 785.7; m p. 268–269°C.

II. N-Methyloxyacanthine: Molecular weight 638.7. Used as the diiodide, molecular weight 892.6; m.p. 258–261°C.

EXPERIMENTAL PROCEDURE. *Rabbits.* Solutions containing 0.25 mgm. of curariform ion per cc. were injected in 15 seconds into a marginal ear vein of each of 24 rabbits (1.8–2.4 kgm.); these were restrained individually in an enclosed box. The doses producing head-

¹ Also called hydroxyacanthine.

² This research was supported in part by a grant from S. B. Penick & Co., New York. We are grateful to Dr. W. G. Bywater, S. B. Penick & Co., New York, for generously supplying "western barberry" root from which the oxyacanthine was isolated by the method of Späth (Ber. 58: 2280, 1925). We are grateful to Dr. D. L. Tabern, Abbott Laboratories, North Chicago, for generously supplying the d-tubocurarine chloride, and to Dr. E. B. Tucker for technical assistance. After this research was completed, the preparation of N-methyloxyacanthine bromide was accomplished. With due allowance for the difference in molecular weight of the halide salts, this compound was identical in activity with the iodide.

drop lasting a minimum of 3 minutes in half a group of 8 animals were determined (see table 1). The experiments were repeated on the same animals after a one-week interval. After an additional 4-day rest period, the 2-day head drop cross-over assay of Holaday (4) was performed. In this procedure, the rabbits are tied belly down and 0.1 cc. of solution administered intravenously every 15 seconds until the head will not rise when the shaven back is electrically stimulated. Since this is an assay requiring a positive response in all animals, the individual variation introduced is fairly wide; consequently, 0.15 mgm of d-tubocurarine chloride pentahydrate per kgm. was considered one unit, and the data for the N-methyloxyacanthine was adjusted to this point, depending on the response of the individual rabbit on successive days. The results of the 24 pairs of data are given in table 1. Doses 50 and 100 per cent larger were given to each of 2 groups of 10 rabbits, and the LD₅₀ was calculated by the method of Miller and Tainter (5).

TABLE 1

	D-TUBOCURARINE	N-METHYLOXYACANTHINE
Albino rats		
LD ₅₀	0.22 (0.27)*	0.43 (0.60)
Rabbits		
Head drop 50.	0.10 (0.12)	0.13 (0.18)
Holaday head drop	0.12 (0.15)	0.15 (0.21)
LD ₅₀ ..	0.19 (0.24)	0.24 (0.33)
Dog		
Gastrocnemius muscle equivalent paralysis.....	0.06 (0.075)	0.11 (0.15)
Head drop... ..	0.13 (0.16)	0.20 (0.28)
Man		
Head drop (3 minutes).. ..	0.12 (0.15)	0.23 (0.32)
(9 minutes).... ..	—	0.27 (0.375)
(18 minutes).. ..	0.16 (0.20)	—

* All doses given in mgm. of curariform ion per kgm. body weight. Dose of equivalent amount of salt used given in parenthesis.

The animal data were treated by the method of Miller and Tainter (5). The standard errors of all the rat data lie within 6 per cent of the figures given; within 5 per cent for the rabbit data, and within approximately 9 per cent for the dog data. In spite of the narrow limits of the calculated standard errors, there is a variation in these data for d-tubocurarine chloride and that of a previous publication (6).

Isolated sections of jejunum from 2 rabbits were prepared for recording by the usual Magnus technic in Tyrode solution and the responses to acetylcholine chloride and to histamine phosphate were tested before and after addition of the curariform agents.

Rats. The relative toxicity was determined in 60 albino male rats (170-250 grams). Solutions containing 0.10 mgm. of curariform ion per cc. were injected intraperitoneally and the lethal doses were determined (see table 1). Four to 10 minutes after receiving a lethal dose, the animals became limp and unable to walk, respiration stopped in an additional 3 to 6 minutes, and finally cardiac activity ceased. The rats did not show any gross signs that these agents had cholinergic activity. They did not sneeze or salivate or evidence chromodachyria or flush as they do with some samples of crude curare.

Dogs. Since cats were unavailable, the gastrocnemius muscle preparation was performed on dogs. Three hundred mgm. of sodium barbital per kgm. were administered intraperitoneally 90 minutes prior to operation in 6 dogs (5.7-8.4 kgm.). The femoral and sciatic

nerves to one leg were cut. The peripheral end of the cut sciatic nerve was stimulated for one-tenth second with 6 volts 60 cycles half wave every ten seconds by a motor driven interrupter. The contractions of the gastrocnemius muscle were recorded on a soot kymograph with a weighted lever. Carotid blood pressure was recorded with the usual mercury manometer. The dogs were mechanically oxygenated.

Fifty and 75 micrograms of d-tubocurarine chloride pentahydrate per kgm. were given intravenously as reference paralyzing doses. The dogs were apparently slightly less sensitive to d-tubocurarine than the cats used in a previous problem (6) since the larger dose was

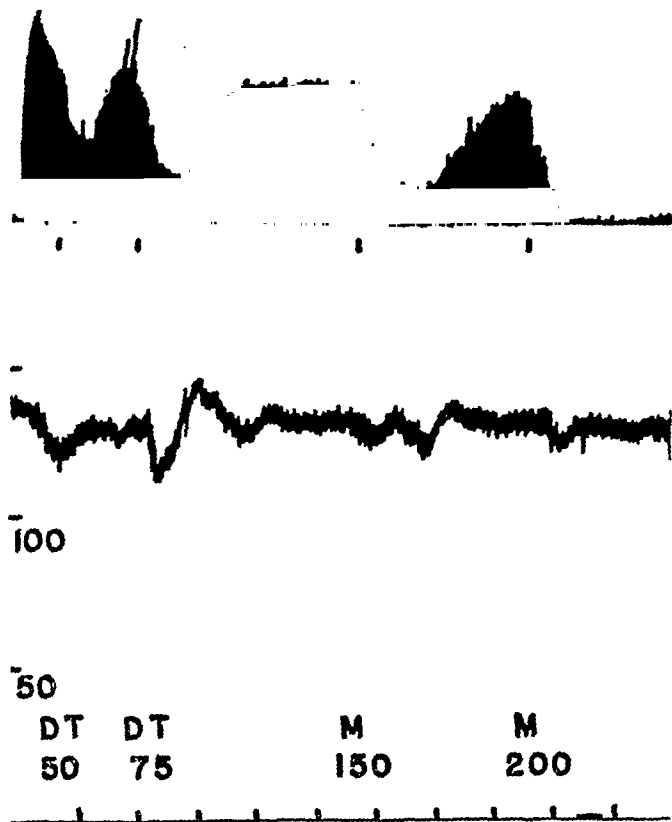


FIG. 1. 6.3 KGm. MALE DOG. GASTROCNEMIUS CONTRACTIONS, ABOVE, AND BLOOD PRESSURE, IN MM. HG, BELOW

Time marks at 5-minute intervals. Fifty micrograms d-tubocurarine chloride pentahydrate per kgm. given at DT/50; 75 micrograms at DT/75. One hundred fifty micrograms N-methyloxycanthine iodide per kgm. given at M/150; 200 micrograms at M/200.

required to produce the expected amount of paralysis. After the muscle returned to normal, various doses of the other agents were given until equivalent paralysis was produced (see figure 1). The muscle could be partially paralyzed by this procedure for 4 to 8 times before contractility no longer returned to normal. Recent experiments indicate that the effects produced in a given animal by 65, 75, and 85 micrograms of d-tubocurarine chloride per kgm. differ by at least 5 per cent.

Four additional dogs were anesthetized with sodium baribital and were prepared for blood pressure recording. These animals received small doses of acetylcholine chloride and epinephrine hydrochloride before and after partially paralyzing doses of the curari-form agents (see figure 2).

Modified head drop cross-over assays were performed in 3 trained, female litter-mate, pedigreed dogs (Boxers: 17-18 kgm., 10 months old). The agents (0.25 mgm of curariform ion per cc.) were administered intravenously in 30 seconds or less into the minor saphenous vein while the dog reclined on one side. The animal was allowed to rise, and the time of onset of paralysis (inability to stand) and its duration were recorded with any other objective findings. Doses of agents producing paralysis lasting 3-10 minutes could be readministered on the following day and the same duration of action, within ± 45 seconds, could be obtained. These assays with unanesthetized dogs were introduced as a preliminary to the investigations in man.

Man. In order to have information concerning possible clinical findings with N-methyloxyacanthine iodide, two experiments were carried out on one of us (D. F. M.) who had been previously standardized to d-tubocurarine chloride (6). The 80 kgm. 29-year old white

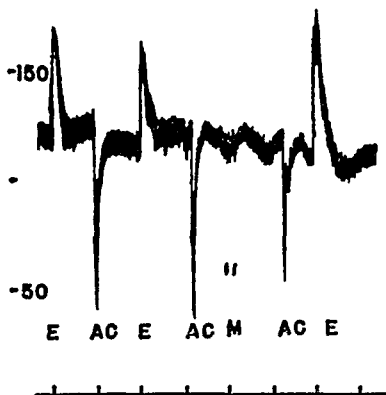


FIG. 2. 7.0 KGm. MALE DOG. 330 MGm. NA BARBITAL PER KGm.

micrograms)
AC; and 150
ve M. Rec-

male was placed in a modified Fowler's position on a treatment table (back elevated about 40° from the horizontal, legs about 120° to trunk). A strap was placed around the lower thighs to prevent the subject from slipping off the table. The N-methyloxyacanthine iodide was given intravenously over a two minute period as a 0.5 per cent solution. The outstanding features of the effects from one experiment are given in figure 3. Other than the occurrence of dryness of the mouth and nose, effects were similar to those noted with d-tubocurarine chloride (6).

RESULTS. Depending on the test procedure adopted, the N-methyloxyacanthine is one-half to three-fourths as active a paralyzing agent as d-tubocurarine chloride (based on molecular equivalence; slightly smaller ratios are obtained if the particular salts actually used are considered).

Although these two agents are primarily curariform in action, they differ slightly in side-effects. In our experiments with isolated rabbit jejunum, we found that as much as 800 mgm. of d-tubocurarine chloride per liter had no

apparent effect on the response of the gut to acetylcholine (1:30 million) or histamine, although 40 mgm. of N-methyloxyacanthine per liter blocked from 50 to 75 per cent of the effect of acetylcholine, but had little or no effect against the histamine. Similar effects were produced by 2 to 4 micrograms of atropine sulfate per liter. Similar lack of activity was found for d-tubocurarine chloride by McIntyre (7) although Gross and Cullen (8) found 'Intocostrin' to have atropine-like effects on human intestine.

Neither of these two agents has any obvious cholinergic or histaminic effects in intact rabbits or rats. In trained dogs, paralyzing doses of d-tubocurarine

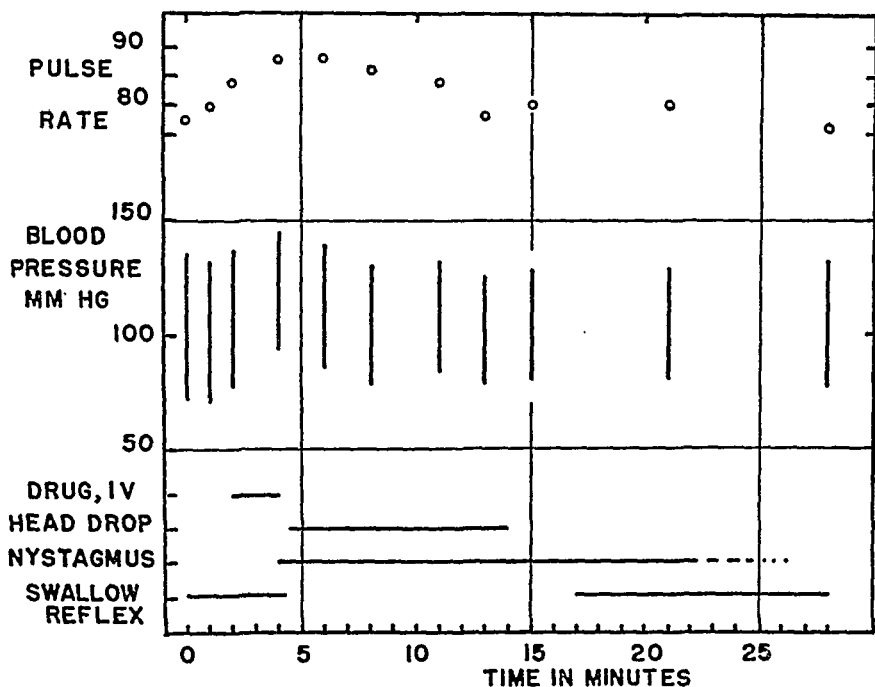


FIG. 3. HUMAN MALE (80 KGm.). THIRTY MGm. OF N-METHYLOXYACANTHINE IODIDE (0.5 PER CENT SOLUTION) WERE GIVEN INTRAVENOUSLY AT TIME INDICATED

Pulse rate, beats per minute, above; systolic and diastolic blood pressure, in mm. Hg, between; and presence of miscellaneous effects indicated by solid lines below.

chloride produce copious salivation; paralyzing doses of N-methyloxyacanthine iodide produce no salivation. A somewhat similar situation exists in man: the subject complained of the inability to swallow what he felt was the accumulation from normal salivation and the material had to be swabbed out by one of the operators periodically during the d-tubocurarine head-drop experiments; during the experiments with N-methyloxyacanthine the subject experienced a dryness of the mouth which persisted for several hours after the experiment had terminated and no swabbing was necessary.

In the anesthetized dog, d-tubocurarine chloride had no particular effects on the response to acetylcholine and epinephrine, although N-methyloxyacanthine

partially antagonized acetylcholine and slightly potentiated epinephrine (figure 2).

DISCUSSION. Brown and Fraser (9) discovered that almost any compound possessing a quinquivalent nitrogen atom within its molecule possessed some curariform activity. However, for a compound to be clinically useful as a curariform agent, either it must be specific enough that it does not produce any undesired side-effects or else it must combine two desired effects. Of the many quaternary ammonium compounds with curariform activity, only those with the d-tubocurarine type of molecule (I) have been sufficiently free of side-effects to be accepted clinically. Although d-tubocurarine is extensively used in anesthetic medication for relaxation of the abdominal musculature, it has not gained universal acceptance for use in convulsive and spastic states, whether these be the result of therapy (metrazol, electroshock) or disease and accidents. The introduction of an agent, such as N-methyloxyacanthine, which produces the desired skeletal muscle relaxant effect and yet does not produce the danger of laryngospasm from aspirated saliva, might lead to greater adoption of this type of therapeutic agent.

In an earlier paper (6), it was demonstrated that quaternary ammonium compounds which possessed the typical bis-1-(p-oxybenzyl)-6,7-dialkoxytetrahydroisoquinoline molecule had curariform activity without appreciable side activity. N-Methyloxyacanthine has virtually the same structural components except that they have been combined with the isoquinoline rings adjacent (II) instead of being alternated with the two oxybenzyl groups as in the d-tubocurarine molecule (I). This interchange of two rings has led to a slight reduction in activity (although several agents with the other arrangement are even less active (6)) and has added a weak atropine-like activity to the molecule.

SUMMARY

N-Methyloxyacanthine iodide was compared with d-tubocurarine chloride pentahydrate in rats, rabbits, dogs, and man. N-Methyloxyacanthine is about one-half as active a paralyzing agent as d-tubocurarine, but differs in that it also possesses a weak atropine-like action.

REFERENCES

1. HESSE, O.: *Ber deut. chem. Ges.*, **19**: 3190, 1886.
2. VON BRUCHHAUSEN, F., AND SCHULTZE, H.: *Arch. Pharm.*, **267**: 617, 1929.
3. KING, H.: *J. Chem. Soc.*, **1948**: 265.
4. VARNEY, R. F., LINEGAR, C. R., AND HOLADAY, H. A.: *Fed. Proc.*, **7**: 261, 1948.
5. MILLER, L. C., AND TAINTER, M. L.: *Proc. Soc. Exper. Biol. and Med.*, **57**: 261, 1944.
6. MARSH, D. F., SLEETH, C. K., AND TUCKER, E. B.: *THIS JOURNAL*, **93**: 109, 1948.
7. MCINTYRE, A. R.: *Curare*, University of Chicago Press, 1947.
8. GROSS, E. C., AND CULLEN, S. C.: *Anesthesiology*, **6**: 231, 1945.
9. BROWN, A. C., AND FRASER, T. R.: *Proc. Roy. Soc. Edinburgh*, **6**: 557, 1869.

THE EFFECT OF ANESTHETICS ON THE UPTAKE OF RADIOACTIVE PHOSPHORUS BY HUMAN ERYTHROCYTES¹

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The uptake of phosphate by the red blood cells has been the subject of many investigations although only a few determinations have been made using radioactive tracers. The migration of phosphate between plasma and cells was studied by Halpern (1). Her conclusions were that at 3°C. the membrane is not permeable to inorganic phosphate, at 23°C. slowly, while at 37.5°C. the transfer is easily demonstrated. Variations in carbohydrate metabolism were induced *in vitro* by the addition of glucose and sodium fluoride, and the subsequent changes in the distribution of phosphate were then observed. Inorganic phosphate entered the cell during glycolysis but left the cell when glycolysis was completed even though its concentration was already higher in the serum than in the cells. Eiseman, Ott, Smith, and Winkler (2), using radioactive phosphorus, confirmed the observations of Halpern. They found that inorganic phosphorus is excluded from the cells at 7°C. and freely enters the cell at 38°C. They believe that some inorganic phosphate is converted into an organic form. The mechanism of entry of phosphate into tissue cells has been reviewed recently by Sacks (3). In view of these experiments and the interest in the effect of anesthetics on cellular processes, it was decided to use the transfer of radioactive phosphate from plasma into the red blood cell as a means of studying the effects of anesthetics on this enzymatic transfer mechanism.

Early in the investigation, it was found that considerable variations exist in the uptake of phosphate by various bloods. Even the same individual does not show the same degree of penetration from day to day. Therefore, this research was extended to cover a study of thirty individual blood donors. Some of these individuals were tested repeatedly from day to day under uniform and also varied conditions of food intake.

METHODS. Human blood was used in this investigation. In some preliminary experiments it was oxalated (200 mgms. of potassium oxalate per 100 cc. of blood), but the majority of experiments were carried out with citrated blood using the ACD² preservative. One-hundred cc. of blood thus contained 1.91×10^{-3} moles of citrates at pH 6.8-7.0 and 600 mgms. of dextrose. In these latter experiments bloods of individual donors were used. When drawn in the morning the blood was used immediately; when drawn in the afternoon it was kept at 4°C. overnight.

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² ACD preservatives: Trisodium citrate, 166.2 grams; citric acid, 59.50 grams; dextrose, 300 grams. Added to 5000 cc. distilled water. Filtered and 50 cc. added to each 500 cc. blood.

A ten cc. specimen of blood was placed in a test tube (20 X 140 mm.). If necessary, the drug, in the form of powder, was added and dissolved. One cc. of radioactive phosphate in 0.9 per cent NaCl solution was then added, the tubes closed and rotated at a rate of 90 rpm at 37°C. Control experiments without the drug were carried out in all determinations. After various periods of time specimens were taken, centrifuged, and the plasma used for determination of radioactivity. Simultaneously, hematocrit readings were taken to trace any possible variation in blood cell volume. Hematocrit readings were made after 30 minutes centrifugation at 1700 rpm.

For the determination of the amount of radioactivity, the following technique was found to give reproducible results. Standard aluminum dishes (supplied by one of the manufacturers) were flattened by means of an hydraulic press. The dishes were washed carefully with alcohol and ether. By using a rubber stamp with a metal ring as a guide, a ring of silicon grease was stamped in the middle of each dish. Then a 0.2 cc. sample of

TABLE I

The effect of mass and the amount of plasma on the determination of radioactivity in plasma (per 0.2 cc. of plasma)

Ashing the samples

SAMPLE NO.	MINUTES FOR 2048 COUNTS	
	Dry	Ash
1	1.45	1.45
2	1.67	1.70
3	1.75	1.76

Varying the amount of plasma

SAMPLE NO.	PLASMA	COUNTS PER MINUTE	COUNTS PER 0.1 CC.
	cc.		
1	0.1	310	310
2	0.2	580	290
3	0.3	890	297
4	0.4	1130	283

plasma was placed in the middle of the ring and with the help of a fine copper wire and a trace of di-octyl-sodium-sulfo-succinate the plasma was spread evenly within the ring. The sample was dried at 80°C. To determine the radioactivity of the solution added to the blood, 1.00 cc. of radioactive phosphate was added to 10 cc. of water, and 0.2 cc. of this solution was used for the determination of the radioactivity of the original phosphate solution.

All measurements of radioactivity were carried out by determining the time required for 2048 counts to take place. This operation was repeated ten times. In consequence, every experimental point was based on 20,480 counts. The results were corrected for the background counts which were generally at the rate of 30 to 40 per minute. The over-all average experimental error was about ± 1.5 per cent.

Two factors may interfere with the validity of the measurements. The first is self-absorption: the absorption of beta radiation by the sample itself. This factor was tested by ashing of the plasma and also by increasing the volume and, therefore, the weight of the sample. In table I are given the results of these experiments. Examination of this table shows that ashing does not change the time necessary for 2048 counts. The same is true, up to a certain limit, when the amount of plasma is varied. Therefore, self-absorption in these experiments is negligible.

Another variable in these experiments is the possible effect of carbon dioxide and oxygen on the penetration of radioactive phosphate. In table II are given the results of an experiment in which the same blood was saturated with air and with a mixture of 5 per cent carbon dioxide and 95 per cent oxygen. Surprisingly, these various bloods showed identical counts within the experimental error. The rate of penetration of radioactive ions is not affected by these gases under the conditions of these experiments. Therefore, it was decided to carry out all the determinations in atmospheric air. It is of interest to note that the absence of oxygen does not seem to affect this rate of penetration (table II).

In order to obtain the magnitude of the exchange of radioactive phosphate between cells and plasma the following determinations were made: 1. the total radioactive phosphate added to blood; 2. the amount of radioactive phosphate in an aliquot part of plasma; and 3. the volume of plasma (hematocrit readings). The number of counts and, therefore, the

TABLE II

The effect of saturating citrated blood with various gases at 37°C.

TREATMENT	COUNTS PER MINUTE PER 0.2 CC. OF BLOOD:			
	After 1½ hrs.		After 3 hrs.	
	In plasma	In blood corpuscles	In plasma	In blood corpuscles
Saturated with air.....	1070	820	750	1000
Sat. 5% CO ₂ : 95% O ₂	1000	890	770	980
Saturated with N ₂	1040	850	760	990
Saturated with N ₂	1010	880	740	1010

concentration of radioactive phosphate in plasma or cells can easily be calculated from the following equations:

$$\text{Counts in 0.2 cc. plasma} \times \frac{\text{Per cent of plasma}}{100} = \text{Counts in plasma per 0.2 cc. of blood}$$

$$\text{Counts in plasma} \frac{\text{per 0.2 cc. of blood}}{\text{Counts in 0.2 cc. of blood}} \times 100 = \text{Per cent of P 32 in plasma}$$

$$100 - \text{Per cent of P 32 in plasma} = \text{Per cent of P 32 in blood cells}$$

RESULTS. Considerable variation from individual to individual was found in the rate of penetration of radioactive phosphate ions into the cells. The frequency distribution diagram of 27 blood donors is given in fig. 1. The spread of experimental points is symmetrical after 1 hour and is less after 3 to 4 hours. After 6 hours, the segregation of experimental points was much better (not reproduced on the graph). This is probably due to the fact that the system approached an equilibrium.

The variation between individual blood donors led to the study of the variation of the rate of penetration of P 32 in a single individual under controlled conditions. J. L., a healthy, athletic, young man was asked to eat an average lunch between noon and 1:00 p.m. At 3:00 p.m. 50 cc. of his blood were drawn. The operation was repeated daily for 6 days. The results are given in fig. 2. The blood of J. L. showed considerable variability, equal, if not greater, than the variability between individual blood donors. Particularly striking are the results of two experiments when in 1 hour practically 80 per cent of P 32 was

transferred into the cells. The only noticeable difference which the blood showed on those days was a slight milky-white appearance. In consequence, it was thought that an acceleration of penetration of P 32 may have been due to the presence of fats or lecithin.

In order to clear up that point, it was decided to restudy an individual donor. This time, R. G. was requested to abstain from breakfast on 2 days (the blood was drawn at 9:00 a.m. and used immediately). On 2 other days, he was asked to eat a copious breakfast (8:00 a.m.), drink 2 glasses of milk and eat a large amount of butter. At 10:30 a.m., 50 cc. of his blood were drawn and used immediately for experiments. The results of these four experiments are given

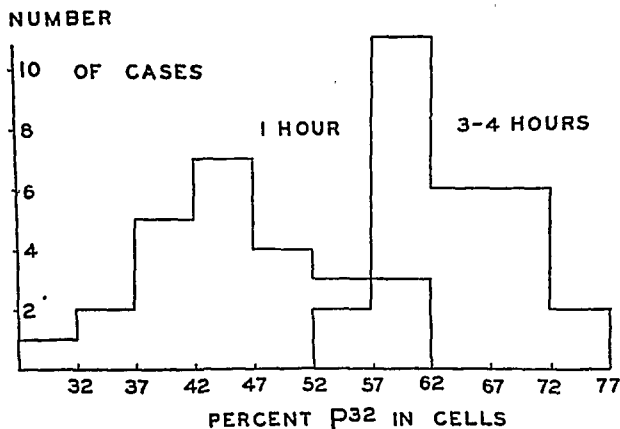


FIG. 1. RATE OF PENETRATION OF P 32 INTO HUMAN ERYTHROCYTES

Frequency distribution diagram of 27 individual blood donors. Citrated blood at 37°C. Abscissae, per cent P 32 in cells; ordinates, number of cases. Results are given for the determinations at the end of one hour and for the 3 to 4 hour determinations.

in fig. 3. Again this system demonstrated the same uncontrolled conditions. The food intake does not seem to be a governing factor in the rate of uptake of P 32. The two experiments before breakfast showed a wide gap at 1 hour. At 6 hours, the system showed a far more orderly behavior and the two sets of experiments exhibited a good segregation. The bloods before breakfast had about 60 per cent of P 32 in the cells; the bloods after breakfast, about 76 per cent.

The average curves for citrated blood (27 individuals) and for oxalated blood (10 experiments) are given in fig. 4. On the same graph is plotted the results of experiments with citrated blood as a reciprocal of time. This straight line was extrapolated to infinite time, indicating that at equilibrium conditions, 70 per cent of P 32 is in the cells.

The variability of the uptake of phosphate discussed in a previous paragraph

leaves two possible solutions: 1. to make an exhaustive study of a certain drug sufficient to give a probable quantitative solution of the problem, or 2. to make a restrictive study of the effect of a certain drug and be satisfied with the answer "yes" or "no." In the case of sodium barbital, an extensive investigation was carried out covering a large number of individual donors. In the case of ether, methadon, and urethane, only a few experiments were made to determine whether these drugs have an effect on the uptake of phosphate.

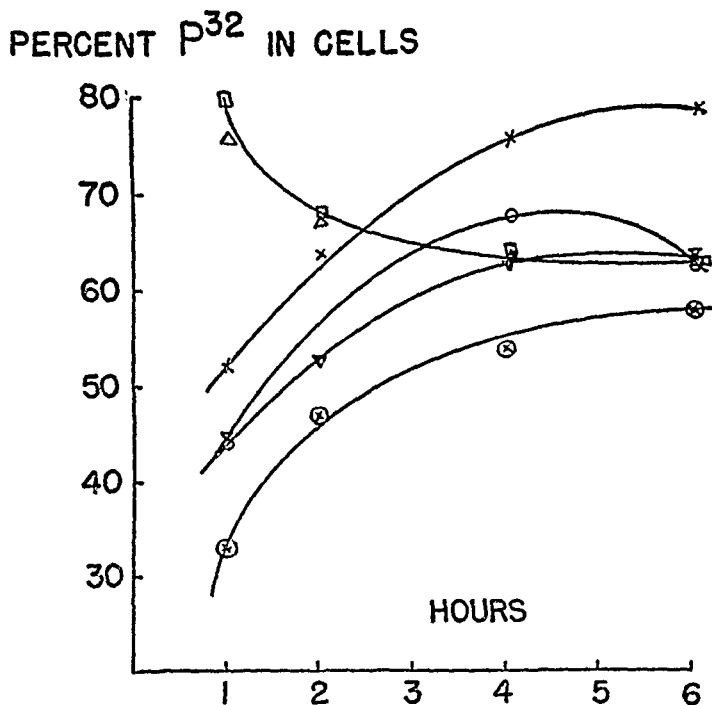


FIG. 2. RATE OF PENETRATION OF P^{32} IN A SINGLE INDIVIDUAL (J. L.)

Six experiments on different days. Citrated blood at 37°C . Abscissae, hours; ordinates, per cent P^{32} in cells.

Sodium barbital, in relatively small concentration, 0.02 Molar, has a distinct delaying effect on the uptake of radioactive phosphate by the cells. Fig. 5 gives the results of these determinations, using the bloods of 21 donors. In the same graph are found the corresponding controls to which no sodium barbital was added. This drug had a very definite effect at the beginning of the experiments, but after 6 hours the sodium barbital curve began to coincide with the control curve due to a higher rate of penetration of radioactive phosphate. It may, perhaps, be said that the mechanisms for uptake have the power of recovering from the initial inhibitory effect and gradually resume a higher state of migration.

Sodium barbital is osmotically active in blood, i.e., sodium barbital does not enter the cells. In table III are found the averages of determinations of the

change in volume of blood cells. Examination of the table demonstrates that the permeability of cells to sodium barbital does not change in the course of the experiment, indicating first, that the cell is not injured by a 6-hour contact with sodium barbital; and second, that the recovery of the cell from initial inhibition is not due to any redistribution of sodium barbital between the cells and plasma. Since sodium barbital is osmotically active, the blood cells lose water. Therefore, it may be suspected that the initial difference (1 hour) may simply be due to

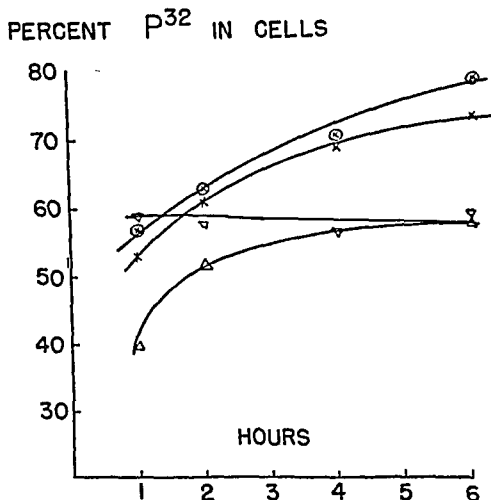


FIG. 3. RATE OF PENETRATION OF P 32 IN A SINGLE INDIVIDUAL (R. G.)

Citrated blood at 37°C. Abscissae, hours; ordinates, per cent P 32 in cells. Upper two curves, X and O, blood samples taken after breakfast; lower two curves Δ and ∇, blood samples taken before breakfast.

a flood of water from the cells into the plasma. This would produce a temporary physical barrier to the migration of radioactive phosphate into the cells.

If this delay is an osmotic phenomenon, then another osmotically active substance should reproduce the delay observed in the case of the sodium barbital curve. In table IV, the results of Exp. 50 are given in detail. In this experiment, sucrose was used as the osmotically active substance. The results do not leave any doubt concerning the specific effect of sodium barbital. In an iso-osmotic concentration, sucrose does not delay the penetration of radioactive phosphate into the cells. It must be concluded that the effect of sodium barbital is a true change in the mechanisms of uptake of phosphate. In order to produce the same amount of shrinkage in cells, the concentration of sucrose required is

Urethane was tried at concentrations of 0.02 and 0.04 Molar. The experiments extended from one to six hours. Beside the usual controls, experiments were done with urea at the same concentration as urethane. Urethane is osmotically inactive in blood; that is, it enters the cells. The results of four experiments indicate that neither urethane nor urea have any effect on the uptake of phosphate ions by the red blood cells.

DISCUSSION. In this study of the effects of certain anesthetics on cellular permeability, the system plasma-red blood cell was chosen on account of its ease of study. However, this simple system presented many difficulties in experimental analysis. Due to the variability in the rate of uptake of phosphate from day to day of blood from different donors and even from the same donor, each experiment had to be evaluated by itself. Fortunately, this could be done by using the same blood sample with and without the anesthetic present. Therefore, the blood sample in each determination had the same amount of citrate

TABLE IV
*Effect of sodium barbital and sucrose on cell volume and uptake of P 32
by red blood cells at 37°C. (citrated blood)*

HOURS	CELLS BY VOLUME			P 32 IN CELLS		
	Control	0.04 M sucrose	0.02 M Na barb.	Control	0.04 M sucrose	0.02 M Na barb.
	%	%	%	%	%	%
1	37.9	34.9	34.7	50.9	52.6	39.0
3	38.2	34.7	35.1	61.5	62.6	52.7

and glucose and was handled in the same manner, the only difference between samples being the presence or absence of the drug in question. There can be no doubt that sodium barbital in the concentrations used decreases the rate of uptake of radioactive phosphate by the red blood cell. Ether also has a retarding effect. Methadon (in very dilute solutions) and urethane do not effect the rate of uptake of phosphate by the red blood cell.

From the fact that the rate of uptake of phosphate is abolished by a decrease in temperature and is dependent on the concentration of glucose in the plasma, it is obvious that the change in uptake is not merely one of change in permeability of the red cell membrane but depends on several factors. The chief factor must be an enzymatic action involving the formation of hexose-phosphates in the cell or on the cellular membrane. Therefore, it is probable that the anesthetics which delay the uptake of phosphate are acting on an enzymatic process.

SUMMARY

1. The rate of uptake of radioactive phosphate ions by human erythrocytes is a variable. It varies from individual to individual and in the same individual from day to day. This change seems not to be a function of food intake.
2. Sodium barbital decreases the rate of uptake of radioactive phosphate ions

by the red blood cell. Certain quantitative conclusions were derived from experiments covering a large number of individual blood donors.

3. Ether has a retarding effect. Methadon (in very dilute solution) and urethane do not change the rate of uptake of phosphate ions by the red blood cell.

REFERENCES

1. HALPERN, L.: J. Biol. Chem., **114**: 747, 1936.
2. EISEMAN, A. J., OTT, L., SMITH, P. K., AND WINKLER, A. W.: J. Biol. Chem., **135**: 165, 1940.
3. SACKS, J.: Chem. Rev., **42**: 411, 1948.

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	Control	0.04 M sucrose	0.02 M Na barb.	Control	0.04 M sucrose	0.02 M Na barb.
	%	%	%	%	%	%
1	37.9	34.9	34.7	50.9	52.6	39.0
3	38.2	34.7	35.1	61.5	62.6	52.7

and glucose and was handled in the same manner, the only difference between samples being the presence or absence of the drug in question. There can be no doubt that sodium barbital in the concentrations used decreases the rate of uptake of radioactive phosphate by the red blood cell. Ether also has a retarding effect. Methadon (in very dilute solutions) and urethane do not effect the rate of uptake of phosphate by the red blood cell.

From the fact that the rate of uptake of phosphate is abolished by a decrease in temperature and is dependent on the concentration of glucose in the plasma, it is obvious that the change in uptake is not merely one of change in permeability of the red cell membrane but depends on several factors. The chief factor must be an enzymatic action involving the formation of hexose-phosphates in the cell or on the cellular membrane. Therefore, it is probable that the anesthetics which delay the uptake of phosphate are acting on an enzymatic process.

SUMMARY

1. The rate of uptake of radioactive phosphate ions by human erythrocytes is a variable. It varies from individual to individual and in the same individual from day to day. This change seems not to be a function of food intake.

2. Sodium barbital decreases the rate of uptake of radioactive phosphate ions

INHIBITION OF SUCCINIC OXIDASE SYSTEM BY MEPERIDINE,¹ METHADON,² MORPHINE AND CODEINE³

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Since the work of Quastel and his associates (1, 2, 3) there has been a growing concept that central nervous system depressants produce their effect by inhibiting the enzymes necessary for the oxidation of carbohydrates. It has been shown that many narcotics inhibit oxygen uptake when glucose, lactate or pyruvate are used as substrate but do not inhibit the oxidation of succinate (3, 4, 5). These and other investigations (6, 7, 8) indicate the dehydrogenase, cytochrome and cytochrome oxidase are relatively narcotic insensitive, whereas flavoproteins or unknown components of the oxidative enzyme system are relatively narcotic sensitive. Methadon, meperidine (demerol) and morphine, though very different in molecular structure, have similar *in vivo* action as analgesics. A recent investigation (8) has shown that morphine in concentrations up to 0.01 *M* does not inhibit glucose oxidation by brain tissue *in vitro* whereas 0.005 to 0.01 *M* demerol and 0.001 to 0.005 *M* methadon do produce inhibition.

The purpose of the present investigation was: (a) to determine the effect of proven analgesics on the succinic oxidase system; (b) if possible to determine what part of the system was blocked and (c) to attempt to correlate *in vitro* and *in vivo* action of the drugs. If the principal site of enzymatic blockage could be established then a biochemical basis would exist for the development of better analgesics and for the more efficient and economical testing of the large number of synthetic compounds being developed as possible analgesic agents. Most recent investigations (9, 10) indicate the succinic oxidase system consists of the dehydrogenase, cytochrome b, an unknown factor, cytochrome c, cytochrome a and cytochrome oxidase. Any observed effect of the analgesics on the oxidation of succinate would be due to its action on one or more of these components.

METHODS. The analgesics used were morphine sulfate USP, codeine sulfate USP, meperidine hydrochloride (Winthrop) and methadon hydrochloride (Lilly). Since there are two molecules of the active alkaloid per molecule of morphine sulfate and codeine sulfate, equal molar concentrations have twice as many active groups as methadon hydrochloride and meperidine hydrochloride.

Beef brain homogenate was used as a source of enzymes. About one hundred grams of brain, obtained within 10 minutes after the animal was slaughtered, were immediately

¹ Ethyl-1-methyl-4-phenylpiperidine-4-carboxylate hydrochloride kindly supplied by Winthrop Chemical Co. as "Demerol".

² 4,4-Diphenyl-6-dimethylamino heptanone, 3-HCl kindly supplied by Eli Lilly & Co. as "Dolophine".

³ This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service.

frozen in a mixture of dry ice and acetone. The frozen brain was then broken into pieces of one to three grams and packed in a 25 mm. x 200 mm. test tube. The test tube was slipped into a quart vacuum bottle, previously packed with about 500 grams of dry ice. When stored in a refrigerator the dry ice will last about one week; the tissue is kept at a very low temperature. There was no significant change in succinic oxidase activity for as long as 30 days. The tissue homogenate was prepared by grinding the frozen brain in cold 0.1 *M* phosphate buffer in a glass grinder (11). One part tissue plus 9 parts of buffer (10 per cent homogenate) was used for the Thunberg experiments; a 25 per cent homogenate was used for Warburg experiments.

Succinic oxidase activity was determined by the method of Schneider and Potter (12). Oxygen consumption was measured in Warburg manometers at 36.3°C. with a gas phase of oxygen. Two control vessels with no drug and four vessels with increasing drug concentrations were used in each experiment and served as checks on each other. The contents and concentration of the substances in the Warburg vessels are given with the results for each experimental condition. Oxygen was passed through the vessels 10 minutes followed by a 10 minute equilibration period. The initial readings were then made, the stopcocks closed and the substrate tipped over into the reaction vessels to start the experiment.

The effect of the drugs on succinic dehydrogenase activity was determined by the rate of methylene blue reduction using the Thunberg method. The tubes and contents were chilled in an ice-water bath and evacuated for 3 minutes with a good water aspirator. Reduction time was determined visually using 90 per cent reduction as the end point. There was a tendency for the highest drug concentration, especially of morphine sulfate, to shift the pH of the buffered solution. A Cambridge glass electrode pH meter was used for pH determinations on the contents of the Thunberg tubes and the Warburg vessels after each experiment. Spectral absorption curves of cytochrome *c* were made with a Beckman model DU spectrophotometer. It could be determined qualitatively whether cytochrome *c* was in the oxidized or reduced state.

RESULTS. The effect of the drugs on oxygen uptake by the succinic oxidase system is shown in figs. 1 and 2. Fig. 1 shows a typical experiment with meperidine. Oxygen uptake is linear with time and there is no significant endogenous activity of the homogenate. The relative inhibition of the succinic oxidase system by morphine, codeine, methadon, and meperidine is shown in fig. 2. The per cent inhibition was obtained by dividing the control vessel oxygen uptake for 60 minutes into the difference between the control vessel and the vessel with drugs. There is a marked inhibition by all the drugs. Part of the inhibition by morphine could be due to the shift in pH which was as much as 0.83 pH units at 4×10^{-2} *M*. The pH could not be held constant at 7.4 since morphine at the higher concentrations would not stay in solution at this pH. Methadon and meperidine gave an even greater inhibition at a very constant pH. The pH with these drugs never varied more than 0.1 units. Repeated experiments with methadon (fig. 2) and homogenates prepared at different times show the experiments and degree of inhibition to be quite constant and reproducible.

Spectral absorption curves made on the contents of the Warburg vessels immediately after experiments showed the cytochrome *c* in the control vessels with a high rate of oxygen uptake was in the reduced state. This was to be expected since succinate in the presence of dehydrogenase readily reduces cytochrome *c*. In the vessels with concentrations of morphine, methadon or meperidine which produced significant inhibition, the cytochrome *c* was in the oxidized form. This indicated the dehydrogenase or some factor necessary for the reduction of cytochrome *c* by succinate was being blocked.

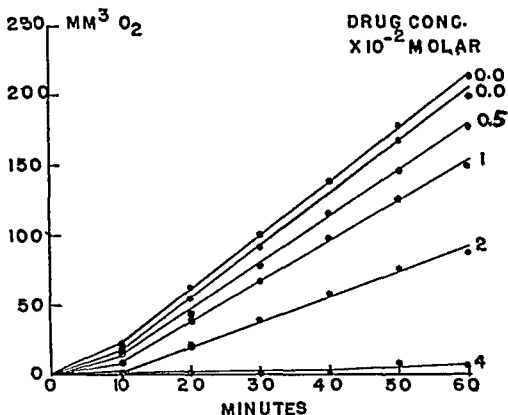


FIG. 1. THE EFFECT OF VARYING CONCENTRATIONS OF MEPERIDINE ON THE SUCCINIC OXIDASE SYSTEM

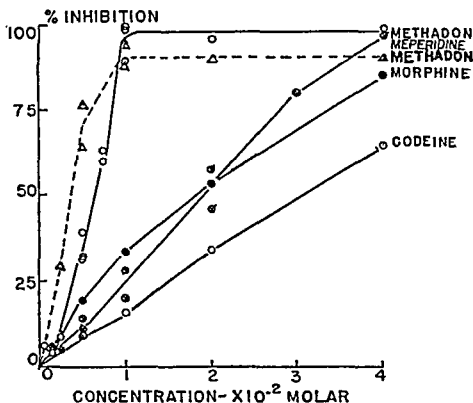


FIG. 2. EFFECT OF VARYING CONCENTRATIONS OF ANALGESICS ON THE SUCCINIC OXIDASE SYSTEM

Solid lines, 0.3 cc. of 0.5 *M* sodium succinate as substrate; broken line, 0.3 cc. of 0.5 *M* *p*-phenylenediamine as substrate; concentration of other components same as for fig. 1.

Since succinic dehydrogenase catalyzes the reduction of methylene blue in the presence of succinate, it was thought this method could be used to determine

the effect of the drugs on the dehydrogenase. The results are given in table I. Morphine inhibited methylene blue reduction. This inhibition is partially due to the decrease in pH which amounted to 0.84 units at 1×10^{-4} *M* morphine sulfate. Time for methylene blue reduction increased from 7 to 27 minutes. In a control experiment when the pH was decreased 0.79 pH units by means of phosphate buffer the reduction time was increased from 18 to 25 minutes. Methadon and meperidine accelerated the reduction of methylene blue by the homogenate both in the presence and absence of succinate. This acceleration is independent of any change in pH which was never lowered more than 0.1 pH

TABLE I

The effect of morphine, meperidine, and methadon on the rate of methylene blue reduction. Temp. 36.3° C.

DRUG	REDUCTION TIME—MINUTES				
	0	0.005	0.01	0.02	0.04
Drug Concentration—Molar.....	0	0.005	0.01	0.02	0.04
Morphine sulfate.....	7	9	11	15	27
Morphine sulfate, no succinate.....	14	40	43	48	60
Meperidine HCl.....	30	26	25	25	18
Meperidine HCl, no succinate.....	101	71	39	23	12
Methadon HCl*.....	16	16	14	7	5
Methadon HCl* no succinate.....	49	34	7	1	1
Methadon HCl, no succinate, no homogenate.....	—	141	27	10	10

Except as noted each Thunberg tube contained 1.0 cc. of 1:10,000 methylene blue; 1.0 cc. of 0.10 *M* phosphate buffer, pH 7.4; 1.0 cc. of 0.15 *M* sodium succinate; drug to give final concentration shown above; water to make 5 cc.; 1.0 cc. of 10 per cent brain homogenate in phosphate buffer in the side arm.

* The methylene blue was in solution with the methadon during a 10 minute equilibration period so that some reduction had occurred before the homogenate was tipped into the tube and the data is only qualitative.

units. Finally it was found that methadon reduces methylene blue in the absence of both succinate and the homogenate. Thus methadon is a reducing agent which requires no biological catalyst to reduce methylene blue. Morphine and meperidine did not reduce methylene blue in the absence of the homogenate. Any observed effect of these analgesics on the rate of reduction would not necessarily be due to their effect on the dehydrogenase.

In a further attempt to isolate the point of blockage by methadon its effect on the oxidation of *p*-phenylenediamine was determined. Only cytochrome *c*—cytochrome oxidase is necessary for the oxidation of this substrate (9). As shown in fig. 2 methadon inhibits the oxidation of the *p*-phenylenediamine even more than the oxidation of succinate. Failure to obtain complete inhibition at the higher concentrations of methadon is probably due to the autoxidation of *p*-phenylenediamine.

DISCUSSION. The analgesics investigated consistently inhibited the succinic oxidase system. The concentration required is about two to five times as great

as that required to inhibit the oxidation of glucose, lactate, and pyruvate by the same analgesics. This is probably the reason most previous investigators have concluded that succinic oxidase is narcotic insensitive. Assuming uniform distribution the concentrations required for *in vitro* inhibition are infinitely greater than that required to produce the *in vivo* effect of the drugs. Though the relative analgesic potency of these drugs has not been firmly established (13, 14, 15), with the exception of meperidine the inhibition produced is in the order of the *in vivo* potency as analgesics.

The attempt to determine the point of blockage by methadon or the other analgesics is inconclusive. Any conclusions to the effect of the drugs on the dehydrogenase would be open to question since other factors effect the rate of methylene blue reduction. The qualitative spectrographic data definitely indicated the dehydrogenase or an unknown factor (9, 10) is blocked, preventing the reduction of cytochrome c in the presence of succinate. Since the oxidation of p-phenylenediamine is also inhibited it must be concluded that methadon also blocks at the cytochrome c—cytochrome oxidase level. This might indicate that the inhibition is not specific but the analgesics at the concentrations used are general enzymatic inhibitors.

The author wishes to express his appreciation to Dr. Chalmers L. Gemmill for his many beneficial suggestions during the progress of this work and to Mrs. R. F. Matthews for her technical aid in the experiments.

SUMMARY

Morphine, codeine, meperidine, and methadon inhibit the oxidation of succinate by brain homogenate. Spectrographic data showed these drugs prevent the reduction of cytochrome c by the enzyme preparation and succinate indicating the block occurred at the dehydrogenase or an unknown factor. The inhibition of the oxidation of p-phenylenediamine by methadon shows blockage also occurs at the cytochrome-cytochrome oxidase level.

REFERENCES

1. MICHAELIS, M., AND QUASTEL, J. H.: *Biochem. J.*, **35**: 518, 1941.
2. QUASTEL, J. H.: *Physiol. Rev.*, **19**: 135, 1939.
3. QUASTEL, J. H., AND WHEATLEY, A. H. M.: *Proc. Roy. Soc. London*, B112: 60, 1932.
4. FUHRMAN, F. A., AND FIELD, J.: *THIS JOURNAL*, **77**: 392, 1943.
5. GREIG, M. E.: *THIS JOURNAL*, **87**: 185, 1946.
6. JOWETT, M., AND QUASTEL, J. H.: *Biochem. J.*, **31**: 565, 1937.
7. JOWETT, M., AND QUASTEL, J. H.: *Biochem. J.*, **31**: 1101, 1937.
8. ELLIOT, H. W., WARRENS, A. E., AND JAMES, H. P.: *THIS JOURNAL*, **91**: 98, 1947.
9. BALL, E. G., ANFENSEN, C. B., AND COOPER, O.: *J. Biol. Chem.*, **168**: 257, 1947.
10. SLATER, E. C.: *Nature*, **161**: 405, 1948.
11. POTTER, V. R., AND ELVEHJEM, C. A.: *J. Biol. Chem.*, **114**: 495, 1936.
12. SCHNEIDER, W. C., AND POTTER, V. R.: *J. Biol. Chem.*, **149**: 217, 1943.
13. SCOTT, C. C., AND CHEN, K. K.: *THIS JOURNAL*, **87**: 63, 1946.
14. SCOTT, C. C., CHEN, K. K., KOHLSTADT, K. G., ROBBINS, E. B., AND ISRAEL, F. W.: *THIS JOURNAL*, **91**: 147, 1947.
15. DENTON, J. E., STRAUS, O. H., WADDELL, W. E., AND BEECHER, H. K.: *Fed. Proc.*, **7**: 214, 1948.

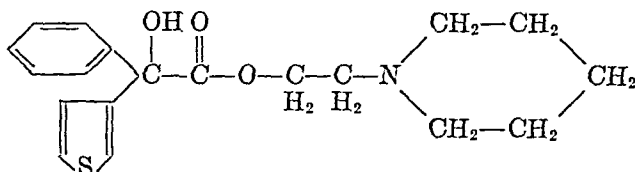
THE PHARMACOLOGY OF β -PIPERIDINOETHYL PHENYL- α -THIENYLGLYCOLATE HCl¹

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A new series of anticholinergic spasmolytic compounds, substituted acetic acid and glycolic acid esters of amino alcohols synthesized by Blicke and Tsao (1), has been investigated recently by Lands and Nash (2) and Lands, Nash, and Hooper (3) for their pharmacologic activity. Preliminary experiments (4, 5) have shown that β -piperidinoethyl phenyl- α -thienylglycolate (PPT)



is most promising and worthy of more intensive pharmacologic study. The work described here was undertaken to determine chiefly the effectiveness of the compound with respect to its spasmolytic action in experimental animals and normal human volunteers, and to determine what side reactions might be expected to occur following therapeutic application to man.

METHODS. 1. *Animal studies.* a) *Cardiovascular action:* Healthy dogs of varying sex and weight were anesthetized with sodium pentobarbital. As an indicator of the anticholinergic effectiveness of this agent, the blood pressure response to acetylcholine before and after the administration of PPT was determined. In order to determine whether PPT possessed any significant antihistaminic action, it was compared with diphenhydramine (Benadryl) employing histamine as the vaso-depressor substance. The intravenous route was employed throughout. Electrocardiographic studies were made in 4 unanesthetized monkeys after subcutaneous administration.

b) *Spasmolytic action:* Effects on the gastro-enteric tract were determined in 17 monkeys pretreated with morphine sulfate, 0.5 to 7.0 mgm./kgm., subcutaneously. The activity of the colon, ileum and jejunum in the acute experiments was measured after an abdominal incision and insertion of single balloons. In the remaining monkeys, the balloons were inserted in tandem into the distal colon by way of the anus and inflated to a pressure which would produce optimal recordings. Air or air-water transmission was used with either Harvard membrane or aneroid manometers. Intestinal activity of the dog was recorded similarly. Normal activity as well as morphine- and pilocarpine-induced hyperactivity were recorded. Intravenous dosages of morphine from 2.5 to 6.5 mgm./kgm. and of pilocarpine 2.0 mgm./kgm. were employed. Effects in the intact uterus were recorded in 4

¹ Aided by a research grant from the Sterling-Winthrop Therapeutic Institute, Rensselaer, New York, which also provided the new drugs used in this study.

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anesthetized non-pregnant multiparous rabbits, using the same recording apparatus as in the dog. The activity of the isolated non-pregnant, guinea pig strip was recorded by the Magnus method using Smith-Ringer's solution. Effects on the intact rat uterus were recorded in 5 anesthetized virgin rats. PPT was administered subcutaneously in all cases.

c) *Chronic toxicity*: Three normal rabbits were given daily subcutaneous doses of PPT, 40 mgm./kgm. until the animals died of convulsions. During the period of this experiment, the sulfobromophthalein retention, blood urea, red and white blood counts, hemoglobin, and differential white cell count were determined every 10 days. Particular attention was paid to the general condition of the animals, appetite, cardiac rate, respiration, size of the pupil and central nervous system irritability. Autopsy was performed immediately after death and sections of the brain, lung, heart, liver, spleen, kidney, adrenal, and intestine were taken for histopathologic examination.

2. *Human studies*. a) PPT was given to normal subjects in doses ranging from 2.5 to 35 mgm. Doses of 2.5, 5, 7.5, 10, and 20 mgm. were administered sublingually; those of 10, 15, 20, 30, 35 mgm., orally. The following observations were made on each subject before and at definite intervals after the administration of PPT:

Central nervous system: Tremors, pupil size, reading ability (as a measure of accommodation), degree of hypnosis (by the number of strokes tapped per minute on a telegraph key), degree of analgesia (by measuring the time of appearance of ischemic pain with a blood pressure cuff applied and pressure maintained over systolic blood pressure).

Autonomic nervous system: Skin: temperature, color, and degree of perspiration.

Cardiovascular system: Pulse rate and character, blood pressure and electrocardiogram.

Respiratory system: Rate and amplitude (by use of the metabor).

b) Gastric motility was recorded in 4 subjects by means of a balloon passed into the stomach and connected by an air system to an aneroid manometer. Doses of 5, 10, and 25 mgm. were administered orally and of 5 and 10 mgm., sublingually.

RESULTS—ANIMAL STUDIES. *Cardiovascular effects. Monkeys*—No effect was noted on cardiac rate, rhythm, or conduction in the monkeys after the subcutaneous administration of 4.0 mgm./kgm. of PPT.

Dogs—The fall in blood pressure from intravenous (i.v.) doses of acetylcholine iodide was effectively combatted by PPT in 14 dogs. The mean fall in blood pressure of two dogs produced by a control dose of 0.001 mgm./kgm. of acetylcholine iodide, i.v., was 59 mm. Hg. After the administration of 10.0 mgm./kgm. of PPT, i.v., graded doses of 0.001, 0.01, 0.1, and 1.0 mgm./kgm. of acetylcholine iodide produced a fall of 4, 16, 51, and 68 mm. Hg, respectively. It is interesting to note that after the administration of PPT, the fall in blood pressure caused by acetylcholine in dosage as large as 1.0 mgm./kgm. (i.e., 52 and 84 mm. Hg) is comparable to that produced by the control dose of acetylcholine, 0.001 mgm./kgm.

In view of the marked anti-acetylcholine effect of PPT demonstrated by this experiment, more extensive studies were instituted in 5 other healthy dogs to determine the intensity and duration of action of this particular agent in smaller doses. Following the injections in the same dog of decreasing doses of PPT, 10.0, 1.0, 0.5 and 0.1 mgm./kgm., i.v., the control dose of acetylcholine, 0.001 mgm./kgm. was repeated at 5 to 10 minute intervals until the blocking action of PPT against the depressor effect of acetylcholine could no longer be demonstrated. Representative records of the decreased blood pressure response to acetylcholine at different intervals after the administration of PPT are illustrated in fig. 1. The results show that PPT in doses of 0.1 mgm./kgm. and up, possesses pro-

nounced anti-acetylcholine activity. The depressor response to acetylcholine, 0.001 mgm./kgm., was completely blocked for considerably long periods by PPT in doses of 0.5, 1.0, and 10.0 mgm./kgm. and remained partially depressed for 71, 124, and 323 minutes, respectively.

In a series of 3 dogs, histamine was given in doses of 0.001, 0.01, and 0.1 mgm./kgm. No effect was evidenced on the histamine-induced fall in blood pressure

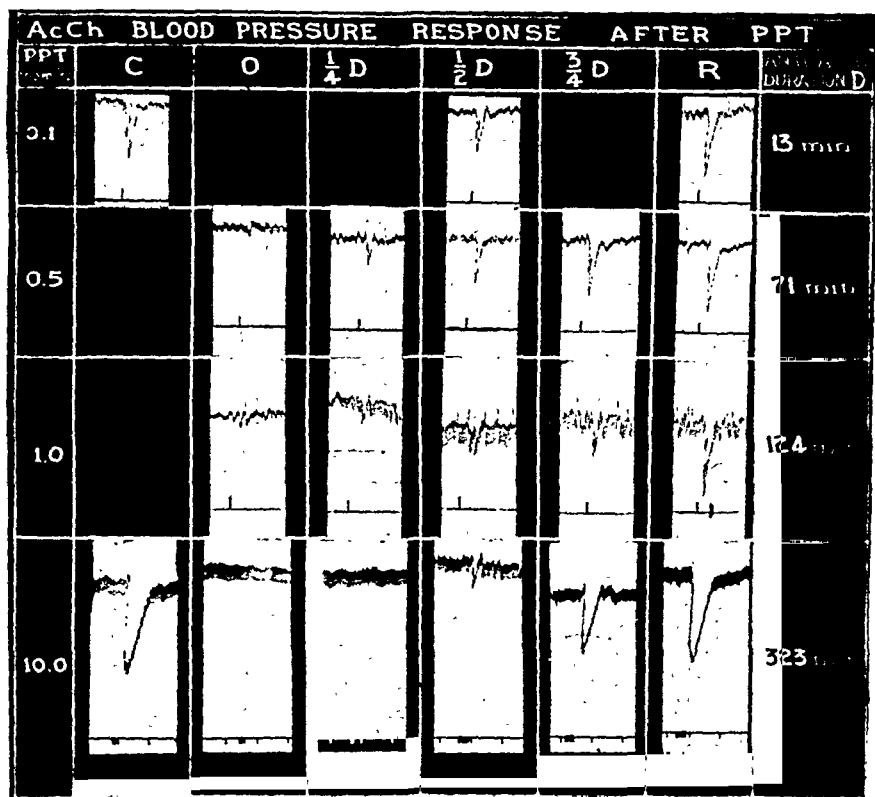


FIG. 1. Duration of anti-acetylcholine effect of PPT on the blood pressure of the dog. Challenge dose of acetylcholine iodide 0.001 mgm./kgm., intravenously, used throughout. C = control response to acetylcholine for PPT doses of 0.1 and 10.0 mgm./kgm. R = recovery response and control response for each subsequent injection of PPT; e.g. R for PPT 0.1 mgm./kgm. is control response for PPT 0.5 mgm./kgm., etc. D = total duration of anti-acetylcholine action expressed in minutes; $\frac{1}{4}$ D, $\frac{1}{2}$ D and $\frac{3}{4}$ D represent responses to acetylcholine injections at time intervals which were $\frac{1}{4}$, $\frac{1}{2}$ and $\frac{3}{4}$ of the total time (D) during which anti-acetylcholine action was present.

after the administration of 10 mgm./kgm. of PPT. There was no demonstrable effect upon the blood pressure rise induced by 0.1 unit/kgm. of pitressin after the intravenous administration of 10 mgm./kgm. of PPT.

Gastro intestinal activity. Monkey—Morphine was given to accomplish a dual purpose in the monkey experiments; first, to induce hypermotility, and second, to quiet the animal during the experimental period. No anti-morphine effect was obtained on the ileum, jejunum, and colon in an acute monkey experiment,

when PPT was administered in subcutaneous doses ranging from 0.1 to 0.4 mgm./kgm. No antispasmodic effect was found in the 7 remaining monkeys

TABLE 1

Duration of spasmolytic action of PPT and Trasentin, subcutaneously, following morphine induced hypermotility of the monkey colon

NUMBER OF MONKEYS	PPT	TRASENTIN	MEAN DURATION AND RANGE OF COMPLETE SPAS-MOLYSIS (MINUTES)
	mgm /kgm.	mgm /kgm	
3	0.4	—	1 (0-3)
2	1.0	—	13 (5-21)
1	1.3	—	3 (1-6)
4	2.0	—	24 (8-52)
4	4.0	—	17 (5-28)
4	8.0	—	42 (5-62)
7		10	4 (0-22)



FIG. 2. INFLUENCE OF PPT AND TRASENTIN ON MORPHINE INDUCED HYPERACTIVITY OF THE MONKEY COLON

Upper record = tracing from upper balloon, lower record = tracing from lower balloon

with subcutaneous doses of less than 0.4 mgm./kgm. In the dosage range of 0.4 to 8.0 mgm./kgm. spasmolytic activity against morphine-induced hypermotility was usually quite marked, especially at the higher dose levels (see table 1).

A representative record is presented in figure 2. Trasentin did not produce spasmolysis until a dose of 10 mgm./kgm. was reached and then only infrequently did it exhibit any action on the monkey colon.

Dog—Results in normal, morphine- and pilocarpine-induced hyperactivity of the ileum and jejunum are summarized in table 2. PPT depressed activity in all of the dogs.

Uterine effects. Rats—No effect could be elicited on the intact rat uterus by PPT in subcutaneous doses ranging from 0.5–8.0 mgm./kgm.

Rabbits—No changes were observed in the intact uterus in doses varying from 1 to 10 mgm./kgm., given intravenously.

Isolated guinea pig uterus—In concentrations ranging from 5×10^{-4} to 5×10^{-2} mgm./cc., the activity was increased from 1 to 4 times that of the control.

Chronic Toxicity. The results of the chronic toxicity study are summarized in table 3.

TABLE 2
Spasmodic effect of PPT in jejunum and ileum of the dog

NO. OF DOGS	PPT mgm./kgm.	RANGE AND AVERAGE DURATION OF DEPRESSION (MIN.)	AGENT USED TO INDUCE ACTIVITY
1	0.2	0	None
1	0.5	34 (30–37)	None
1	2.5	101 (72–130)	None
1	0.5	40	Pilocarpine
1	1.0	58 (55–60)	Pilocarpine
3	0.5	21 (4–30)	Morphine
3	1.0	40 (0–130)	Morphine
1	2.0	52	Morphine

These tests were carried out over a period of 68 days. Slight changes in the blood picture of the tested animals were evidenced by an increase in the number of red and white blood cells.

There was slight impairment of renal and hepatic functions as evidenced by increase in blood urea and sulfobromophthalein retention. All of the animals showed a gradual decrease in body weight and gradual anorexia accompanied by general weakness. Definite mydriasis was regularly shown for about 3 hours following the injection from the second week of administration.

Rabbits No. 1 and No. 2 began to develop mild convulsions on the 44th and 47th day, respectively, and finally succumbed in clonic convulsions on the 46th and 52nd day, respectively. The third animal did not convulse until the 68th day when it died 2 hours after the onset of convulsions.

On autopsy 2 of these animals showed gross signs of congestion of the liver and scattered pale ischemic patches in the kidneys. Microscopic examination of

brain, lung, liver, spleen, heart, intestine, kidney and adrenal revealed no changes which could have been considered as being due to direct pathologic effects of PPT. In the case of one rabbit, early fatty infiltration ascribable to malnourishment was observed and in another there were recent intra-alveolar hemorrhages

TABLE 3

Chronic toxicity in rabbits following the subcutaneous administration of PPT, 40 mgm./kgm. given daily

	RABBIT NO. 1			RABBIT NO. 2			RABBIT NO. 3		
	Con- trol	2nd wk.	6th wk.	Con- trol	2nd wk.	6th wk.	Con- trol	2nd wk.	6th wk.
Body Wt. (kgm.)	3.5	3.8	3.0	4.1	4.3	2.9	3.2	3.0	2.5
Red Cell Count (millions) . . .	5.57	6.32	6.27	6.13	5.37	6.84	4.81	6.71	6.93
White Cell Count (thou- sands)	12.2	14.2	17.7	9.7	11.9	14.8	11.5	18.3	21.7
Differential White Count									
N	75	66	59	71	70	64	69	62	68
L	19	31	36	22	23	33	22	34	26
M	4	3	5	7	6	3	8	4	6
E	2	0	0	0	1	0	1	0	0
B	0	0	0	0	0	0	0	0	0
Sulfobromophthalein % re- tention after 60 min.	17	18	24	10	12	15	20	22	27
Blood Urea N in mgm. % . . .	10.4	12.0	13.6	12.5	14.0	14.7	9.5	12.5	14.0

TABLE 4

Untoward effects of PPT in humans

NO. OF SUBJECTS	DOSE	ROUTE	SIDE EFFECTS
	mgm.		
1	2.5	Sublingual	Local anesthesia at site of application
5	5	Sublingual	Local anesthesia at site of application
3	7.5	Sublingual	Local anesthesia at site of application
2	10	Sublingual	Local anesthesia at site of application
3	10	Oral	No effects
5	15	Oral	No effects
4	20	Oral	No effects
1	20	Sublingual	No effects; local anesthesia at site of application
4	25	Oral	No effects
4	30	Oral	Two subjects were drowsy
4	35	Oral	One subject was drowsy and another, 40 min. after administration, exhibited drowsiness, vertigo, and had dimness of vision and chills lasting 80 minutes

which could be explained as being due to the convulsive asphyxial type of death of these animals.

HUMAN STUDIES. Table 4 summarizes the results obtained in the determination of untoward effects in the human.

The average age of the subjects was 25, ranging from 17 to 35. As indicated in table 4, there were no appreciable effects noted until the 30 mgm. dose level was reached. Only one subject taking a 35 mgm. dose noted marked untoward effects; the first reactions noticed were dimness of vision and malar flushing 40 minutes after the administration of PPT orally; 35 minutes later, the subject had chills; her hands were cold, and forehead skin temperature had dropped 1.8° C. from the value obtained 15 minutes earlier. On standing, she complained that her legs felt "like rubber". No significant changes were noted in the pulse rate, blood pressure or electrocardiogram. The same subject had previously taken 15 mgm. with no untoward effects. Other than the effects indicated, there were no measurable changes observed by the methods employed on the central and autonomic nervous systems, cardio-vascular and respiratory systems.

TABLE 5
Inhibitory effects of PPT on gastric motility

DOSE	ROUTE	NO. OF SUBJECTS	ONSET OF ACTION	DURATION OF ACTION
mgm.			min.	min.
5	Oral	2	—	—
10	Oral	2	—	—
25	Oral	1	—	—
5	Sublingual	1	25	33
5	Sublingual	1	11	19
10	Sublingual	1	5	62
10	Sublingual	1	7	30

Table 5 illustrates the effects of PPT on gastric motility. Sublingual administration of 5 mgm. doses resulted in an inhibition of gastric motility for 19 and 33 minutes and 10 mgm. was spasmolytic for 30 and 62 minutes. Oral administration of 5, 10 and 25 mgm. doses did not inhibit gastric motility.

DISCUSSION. Investigations of agents which possess potent anticholinergic action indicate that when the desired antispasmodic effects are obtained, there will also be some action on effector organs other than those upon which action is desired. Side actions following the administration of a gastro-enteric antispasmodic to the normal human, may imply a host of anticholinergic effects. However, it should be recognized that a cholinergically innervated structure which is in spasm or hyperactive may be responding to a greater than normal amount of acetylcholine. Consequently, it would be reasonable to expect that depression of this type of activity would be possible without marked side effects. It is for this reason that most of the studies which have been conducted in this investigation have been designed to demonstrate drug effectiveness on a spasmogenic background.

PPT is apparently more effective in reducing the morphine-induced hyperactivity of the monkey colon than is trasentin. The preliminary experiments in

human volunteers indicate that PPT is effective in reducing the normal motility of the stomach and suggest that it deserves controlled clinical trial in diseases associated with hypermotility of the stomach and other portions of the gastro-enteric tract.

In the monkeys used, there was no definite effect on the central nervous system in doses as high as 8 mgm./kgm. None of the animals exhibited noticeable side effects upon being released in their cages. However, it has been suggested (6) that the monkey is resistant to some of the peripheral and to the central nervous system effects of these anti-cholinergic compounds.

The central nervous system effects in man were definite only in one subject of four at the highest dose level of 35 mgm. orally. In all probability, this dose approximates the level at which untoward reactions will appear, since four other subjects receiving the slightly lower dose of 30 mgm. did not show any effect.

SUMMARY

1. *The cardiovascular effects of PPT are as follows:* (a) In monkeys, it produces no changes in cardiac rate, rhythm or conduction in subcutaneous doses of 4 mgm./kgm. (b) In dogs, it effectively abolishes the depressor effect of acetylcholine, but in doses as high as 10 mgm./kgm., intravenously, it does not alter the cardiovascular response to histamine or pitressin. (c) In the normal human, doses ranging from 2.5 to 35 mgm. orally, did not produce any changes in blood pressure, cardiac rate, rhythm or conduction.

2. *The gastro-enteric effects of PPT are:* (a) In monkeys, doses varying from 0.4 to 8 mgm./kgm., subcutaneously, induced spasmolysis in the intact colon rendered hyperactive by morphine. (b) In dogs, doses ranging from 1.0 to 2.5 mgm./kgm., intravenously, induced spasmolysis of normally active and hyperactive ileum and jejunum. (c) In the normal human, 5 and 10 mgm. doses, sublingually, depressed normal gastric motility.

3. *The uterine effects of PPT are:* (a) No changes were observed after intravenous doses ranging from 1 to 10 mgm./kgm. in the intact, non-pregnant, rabbit uterus or in the intact rat uterus with subcutaneous doses of 0.5–8.0 mgm./kgm. (b) Increase in activity was noted when concentrations ranging from 5×10^{-4} to 5×10^{-2} mgm./cc. bathed the isolated strip of the non-pregnant guinea pig.

4. *Chronic toxicity:* Daily administration of PPT to rabbits in doses of 40 mgm./kgm., subcutaneously, until death (46–68 days) slightly diminished renal and hepatic functions and increased red and white cell counts. The latter effect may be correlated with a progressive anorexia, weight loss, and dehydration. No significant histopathologic changes were produced.

5. *General effects of PPT in the human:* In doses from 2.5 to 30 mgm., there were no measurable changes in a two-hour observation period on central nervous, cardiovascular or respiratory systems. One subject, of four receiving 35 mgm., experienced vertigo, dimming of vision and chills lasting 80 minutes.

REFERENCES

1. BLICKE, F. F., AND TSAO, M. U.: J. Am. Chem. Soc., **66**: 1645, 1944.
2. LANDS, A. M., AND NASH, V. L.: Proc. Soc. Exper. Biol. and Med., **57**: 55, 1944.
3. LANDS, A. M., NASH, V. L., AND HOOPER, K. Z.: THIS JOURNAL, **86**: 129, 1946.
4. ABREU, B. E., AND CHEN, Y. P.: Unpublished observations.
5. LANDS, A. M.: personal communication.
6. ABREU, B. E., AND Troescher-Elam, E.: THIS JOURNAL, **86**: 205, 1946.

THE ACUTE PHARMACOLOGY OF METHYL-BIS(2-CHLOROETHYL)AMINE (HN2)¹

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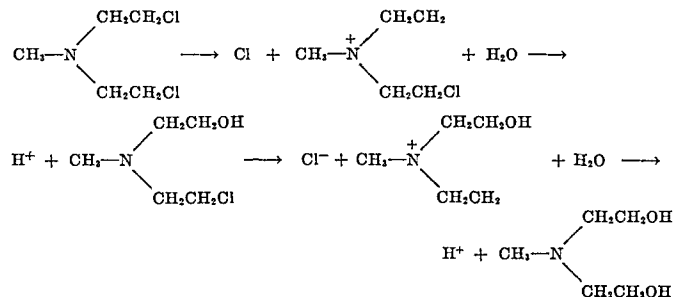
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Recent studies of *bis*(2-chloroethyl) substituted tertiary amines have emphasized their selective actions on proliferative tissues which resemble the effects of penetrating radiations (1). These cytotoxic actions have been used to advantage in the treatment of certain neoplastic diseases (2). The present study, however, concerns the acute pharmacology of methyl-*bis*(2-chloroethyl)amine (HN2) and its active transformation products. In this regard previous investigations of Gilman and Goodman (3) and Smith (4) have shown that HN2 has cholinergic actions, findings which were confirmed and extended by Foss and Gaddum (5). Evans and Foss (6) described the "neurotoxic" effect of certain transformations resulting from the reactions of HN2 in water. Gilman, Goodman, and Philips (7) investigated the pharmacology of products formed by the transformation of HN2 in buffered solutions and related certain of the pharmacologic effects of HN2 to the occurrence of such transformations *in vivo*. In addition, Anslow, Karnovsky, Jager, and Smith (8) investigated the toxicity of crystalline salts of a number of the transformed products of HN2.

METHODS. *Preparation of the transformed products of HN2.* The principal transformations of HN2 in dilute, slightly alkaline, aqueous solutions may be seen in the following series of consecutive reactions:



¹ The work described in this paper was done in part under contract between the Medical Division, Chemical Corps, U. S. Army, and Cornell University Medical College. Under terms of the contract, the Chemical Corps neither restricts nor is responsible for the

Only 3 of the principal stages in the transformation of HN2 have an appreciable existence in mildly alkaline solution, namely: methyl-2-chloroethyl-ethyleniminium (chlorimine), methyl-2-hydroxyethyl-ethyleniminium (hydroxyimine), and methyldiethanol-amine. The chlorhydrin formed by the hydrolysis of chlorimine undergoes rapid cyclization under such conditions. The cyclic chlorimine and hydroxyimine are involved, to a small extent in dilute solutions, in side reactions with different components of the mixture to form various relatively inactive polymers. Details of the reactions described have been fully studied by Bergman and co-workers (9). Advantage was taken of analytic procedures devised by these investigators to obtain solutions which contained as active constituents either the chlorimine or hydroxyimine. For this purpose 0.02M solutions of HN2-hydrochloride² were prepared in 0.16 M NaHCO₃ and maintained at $30 \pm 0.1^\circ\text{C}$. for varying intervals. Analysis of aliquots for Cl⁻ liberated and S₂O₃²⁻ uptake permitted an evaluation of the extent of transformation at any given time.

Solutions obtained after 20 minutes of reaction were found suitable for study of the effects of chlorimine and showed upon analysis 2.00 molar equivalents of Cl⁻ liberated and 1.86 molar equivalents of S₂O₃²⁻ uptake. The analytic results indicate a complete transformation of the parent amine and the presence of chlorimine in solution in an amount equivalent to more than 90 per cent of the initial quantity of HN2·HCl. Chilling in ice water prevented any further significant change in composition for at least 2 hours. Doses of chlorimine were calculated assuming the 20-minute reaction product to contain 1 molar equivalent.

After 15.5 hours of reaction at 30°C ., solutions showed no further liberation of Cl⁻ (2.87–2.91 molar equivalents). The 16.5 hour reaction product combined with 0.42 molar equivalents of S₂O₃²⁻. These results indicate the absence of components capable of cyclization and the presence of hydroxyimine equal to about 42 per cent of the initial amount of HN2·HCl. Chilling in ice water rendered this product stable for hours. Doses of hydroxyimine assumed the presence of 0.4 molar equivalents in such solutions.

After 48 hours of transformation, S₂O₃²⁻ reactivity was reduced to 0.03 molar equivalents. Such solutions were used to confirm previous observations on the relative inactivity of methyl-diethanol-amine and the various dimers formed during the transformation of HN2.

Analysis of transformation products. 1. *Chloride analysis.* Ten cc. aliquots were added to 5 cc. of 0.32 M acetic acid containing 5 drops of 0.1 per cent dichlorofluorescein solution. Titration for Cl⁻ was carried out rapidly with 0.1 N AgNO₃.

2. *Thiosulfate uptake.* Ten cc. aliquots were added to 10 cc. of 0.16 M NaHCO₃ and 10 cc. of 0.1 N Na₂S₂O₃. After reaction at room temperature for at least 2 hours, 10 cc. of 0.32 M acetic acid, 1 gram KI, and 1 cc. of 1 per cent starch solution were added in turn. Thiosulfate uptake was represented by the difference between the iodine (0.1 N) titer of the unknowns and that of 10 cc. of 0.1 N S₂O₃²⁻.

Cholinesterase activity. A modification of the method of Ammon (10) was used. The main wells of Warburg flasks received 3.0 cc. of 1:60 homogenates of salivary glands and 0.5 cc. of water. The side bulbs contained 0.5 cc. of 0.12 M acetylcholine bromide in 0.03 M NaHCO₃. Homogenates were made in the Waring blender, the diluent being 0.03 M NaHCO₃. After gassing with 95 per cent N₂ and 5 per cent CO₂, temperature equilibrium was established at 38°C ., the vessels tipped, and manometric readings taken every 10 minutes. A 30-minute period of constant rate was chosen to calculate activity.

Physiological methods. 1. *Blood pressure.* Adult cats were anesthetized with 30 mgm./kgm. of sodium pentobarbital intravenously and the carotid artery cannulated and con-

opinions or conclusions of the authors. The investigation was also supported by research grants to the Sloan-Kettering Institute from the U. S. Public Health Service, National Institute of Health, Division of Research Grants and Fellowships, and from the American Cancer Society.

² Twice recrystallized from hot acetone; analysis: N, 1.00 equivalent; total Cl, 2.97 equivalents; total S₂O₃²⁻ uptake, 1.99 equivalents.

nected to a mercury manometer. All injections were made into the femoral vein. The right vagus nerve was exposed for stimulation with an inductorium.

2. *Nictitating membrane.* The membrane of cats was connected to an isotonic lever after fixation of the eyeball. The cervical sympathetic trunk was exposed for stimulation. Injections were made into the common carotid artery after appropriate dilutions were made with isotonic NaCl.

3. *Salivary flow.* Adult cats were anesthetized with 0.5 cc./kgm. of "Dial" solution (Ciba) intraperitoneally. The duct to the right submaxillary gland was cannulated and the salivary flow measured by a drop recorder. The *chorda tympani* was cut and there was no spontaneous flow. Injections were made into the right common carotid artery. In certain experiments the right superior cervical ganglion was removed. At the conclusion of these experiments the right submaxillary gland was removed for determination of cholinesterase activity.

TABLE 1

Intravenous toxicity of HN2·HCl and its transformation products in rats

DOSAGE	HN2·HCl		CHLORIMINE		HYDROXYIMINE		48-HOUR PRODUCT	
	Mortality	Time of death	Mortality	Time of death	Mortality	Time of death	Mortality	Time of death
mMols/ kgm.								
0.40							0/6	
0.16	4/4	2 to 4 hrs.	6/6	3 to 4 hrs.	8/8	1 hr.		
0.08	4/4	1 day	6/6	1 day	9/16	1 to 3 hrs.		
0.04	4/4	1 to 2 days	6/6	1 to 5 days	1/6	3 hrs.		
0.02	4/4	3 to 5 days	6/6	3 to 6 days				
0.01	5/6	3 to 5 days	8/8	3 to 6 days				
0.005	6/14	3 to 6 days	2/10	4 to 5 days				
0.0025	1/10	7 days	0/6					

4. *Neuromuscular function.* Isometric recording of the contractions of the intact cat gastrocnemius-soleus preparation was made using a holder described by Wolff and Cattell (11). The tendon was wired to a heavy torsion lever, the popliteal artery exposed for injections, and the sciatic nerve cut and its distal end placed in enclosed silver electrodes. Supra-maximal nerve shocks were delivered by an interruptor from a 1 mfd condenser. The condenser charge was varied by a potentiometer circuit connected to a 45 volt battery. The frequency of stimulation was 1 per 12 seconds.

RESULTS. *Toxicity in rats.* The intravenous toxicity of HN2 and its transformed products was studied in Wistar rats weighing 100 to 250 grams. The toxicity and the survival times following administration of HN2 and its chlorimine were similar, (table 1). Doses less than 0.02 mM./kgm. caused delayed deaths while larger doses killed within 2 to 4 hours and were associated with a progressive muscular paralysis and terminal convulsive seizures. There was little difference in the appearance of rats receiving HN2·HCl and the chlorimine except that the latter sometimes produced a transient prostration immediately following the injection.

The hydroxyimine was less toxic but survival times following lethal doses were

shorter than those following equivalent doses of $\text{HN2}\cdot\text{HCl}$ or its chlorimine. This is in agreement with the studies of Boyland (12) who found that reduction in survival time of mice was related to the extent of hydrolysis of HN2 . Rats which received the hydroxyimine in doses of 0.04 mM./kgm. or greater presented a progressive muscular paralysis which although more rapid in onset, was similar to that seen following HN2 or the chlorimine. Delayed deaths did not occur in animals receiving hydroxyimine. The 48 hour-product was relatively non-toxic and caused no deaths at 0.40 mM./kgm.

TABLE 2
Intravenous toxicity of $\text{HN2}\cdot\text{HCl}$ and its transformation products in cats

DRUG	DOSE	MOR- TALITY	TIME OF DEATH	FLACCID PARALYSIS	PARALYSIS + INCOORDI- NATION	REMARKS
$\text{HN2}\cdot\text{HCl}$	mM./kgm.					
	.08	1/1	<18 hr.	0	+++	
	.04	1/1	<18 hr.	0	+++	
	.02	3/3	1d, 2d, 3d	0	+ -	
	.01	0/3		0	0	2 showed wt. loss
	.005	0/4		0	0	no signs
Chlorimine	.0025	0/2		0	0	no signs
	.02	3/3	15 min.			
			18 hr.	+++	+++	
	.01	3/3	<18 hr.	++	++	
	.005	3/4	1d, 6d, 14d*	0	+ -	
Hydroxyimine	.0025	0/2		0	0	no signs
	.02	3/3	31-50 min.	0	+++	
	.01	3/3	43-86 min.	0	+++	
	.005	3/4	55-75 min.	0	+++	
	.0025	2/6	43-48 min.	0	+++	
	.00125	0/2		0	++	recovery

* Sacrificed.

The results of the present study of the toxicity of HN2 and its transformations is in general agreement with the findings of Anslow, Karnovsky, Jager and Smith (8) who employed the corresponding crystalline salts.

Toxicity in cats. 1. $\text{HN2}\cdot\text{HCl}$. The lowest intravenous dose of $\text{HN2}\cdot\text{HCl}$ which caused immediate effects in intact animals was 0.02 mM./kgm. (table 2). Within 10 minutes after such doses there appeared licking, retching, vomiting, salivation, and frequent, loose stools. Larger doses produced the above signs more rapidly and, in addition, a characteristic neurological disturbance which began within 20 to 30 minutes. Initially the disturbance was indicated by an inability to support the head; this was followed by a progressive paralysis of the entire somatic musculature. The advancing paralysis was associated with gross incoordination, asynergia, kinetic tremor, and dilated pupils which re-

sponded sluggishly to light. Coincidentally there occurred profuse and sustained salivation. The paralysis became maximal within 1 to 2 hours after administration and, thereafter, the animal was prostrate making only abortive movements. Knee-jerks remained active throughout paralysis and no signs of muscular fasciculation or specific disturbance of the placing or righting reflexes was evident. Cats receiving 0.02 mM./kgm. were not paralyzed and succumbed after several days, following progressive weight loss. Convulsions were noted in only 1 cat on the day after administration of 0.02 mM./kgm.

2. *Chlorimine*. Chlorimine produced effects differing from those of HN2·HCl only insofar as its intravenous administration in doses of 0.01 mM./kgm. or more was followed immediately by complete paralysis. During the resulting prostration the skeletal musculature was flaccid and showed widespread fasciculations. Attending the initial paralysis was a brief period of salivation, pupillary dilatation, and bradycardia. Although respiration was stimulated during injection of the chlorimine larger doses were followed promptly by fatal respiratory paralysis. Cats surviving acute effects recovered rapidly and within 5 to 10 minutes walked about in apparently normal fashion.

Subsequently, at approximately 15 minutes after injection of 0.02 mM./kgm. or more of the chlorimine, there developed a second paralysis duplicating in detail that seen after injection of the hydrochloride salt. Inability to support the head was followed by ataxia, incoordination, asynergia, some hypermetria, and a progressive muscular weakness which reached a maximum in about 30 minutes. The delayed paralysis was associated with copious and prolonged salivation and pupillary dilatation. Although improvement of the neurological disturbance occurred, animals succumbed within 18 hours.

The effects of chlorimine differed from those of HN2·HCl principally in regard to the production by the former agent of two clearly separable paralytic disturbances. The chlorimine also caused less retching, vomiting, loose stools, blepharospasm, and ear flicking.

3. *Hydroxyimine*. Within a few minutes after the injection of hydroxyimine cats exhibited a neurologic disturbance which resembled in all respects the delayed paralysis and incoordination following HN2·HCl or its chlorimine. However, severe paralysis followed doses as small as 0.0025 mM./kgm. and consequently the agent proved to be more toxic than either HN2 or chlorimine. Maximal paralysis was obtained from 30 to 40 minutes after administration and was often associated with respiratory arrest. No skeletal muscle fasciculations were noted and salivation was scant in contrast to the copious flow which followed HN2·HCl or its chlorimine. Such animals were similar in appearance to those reported by Evans and Foss (6) after administration of a 24-hour, unbuffered, aqueous hydrolysate of HN2. These investigators in comparisons of the hydrolysate in monkeys, guinea pigs, rats, goats, dogs, cats and a horse found the cat to be most sensitive to its effects while the rat and guinea pig were relatively resistant. The hydrolysate consisted in large part of the chlorhydrin of HN2, the effects of which may be attributed to conversion *in vivo* to the hydroxyimine form.

Administration of hydroxyimine to cats also caused frequent, loose stools for several hours after injection. Cats which survived the paralytic effect of the hydroxyimine appeared normal on the following day and delayed deaths were not observed.

It is interesting to compare the acute actions of HN2 and its transformation products in cats with those noted by Gilman, Goodman, and Philips (7, 13). These workers observed parasympathomimetic effects in rabbits within 5 to 10 minutes after the injection of HN2·HCl which included salivation, defecation, lachrymation, bronchorrhea, and miosis. In addition skeletal muscle tremors and a rise in blood pressure followed intravenous administration in the atropinized cat. Such actions were attributed to muscarinic and nicotinic properties of HN2. It was also noted that chlorimine in rabbits was at least as toxic as the parent compound, evoked parasympathomimetic effects, caused immediate, transient, unexplained prostration, and produced delayed paralysis.

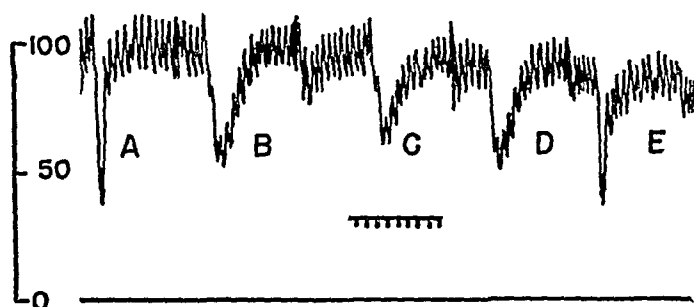


FIG. 1. Carotid blood pressure of cat. A—Vagal stimulation 5 sec., 11:39. B—Acetylcholine, 0.1 μ gm./kgm., i.v., 11:43. C—HN2·HCl, 0.01 mM./kgm., i.v., 11:49. D—Acetylcholine, 0.1 μ gm./kgm., i.v., 11:54. E—Vagal stimulation, 5 sec., 11:59. Time interval 10 seconds.

Hydroxyimine was found to be less toxic than HN2·HCl and less cholinergic than the chlorimine but was strongly paralytic. Large doses of HN2·HCl were shown to be convulsant in rabbits, an action not evoked by equivalent doses of chlorimine or hydroxyimine.

The present study is in agreement with the results obtained in the earlier observations mentioned above. However, in the cat miosis did not follow intravenous administration of HN2 or its transformed products and muscular fasciculations were evident only after injection of chlorimine. The coarse tremor which accompanied the delayed paralysis and incoordination involved whole muscle groups and was probably central in origin.

Effects on blood pressure. 1. *HN2·HCl.* Goodman and Gilman (3) and Foss and Gaddum (5) found that HN2·HCl caused a fall in blood pressure following intravenous administration, an action which was abolished by atropine and replaced by a rise after larger doses. Both groups considered HN2 to have muscarinic and nicotinic effects upon the circulation.

Figure 1 shows the fall in blood pressure following 0.01 mM./kgm. of HN2·HCl

intravenously. The responses to vagal stimulation and to injected acetylcholine were not significantly altered by this dose. Larger amounts (0.02 mM./kgm.) abolished the circulatory response to vagal stimulation but had no significant effect on responses to injected acetylcholine or epinephrine. The vagal blocking action of HN2 is less striking than that of *tris* 2-chloroethyl amine (14). No tachyphylaxis became evident after repeated doses of HN2.

2. *Chlorimine*. When given intravenously chlorimine caused responses similar to those following HN2·HCl but proved to be more potent than the parent amine. Thus 0.002 mM./kgm. was depressor while 0.01 mM./kgm. caused pressor responses without the prior administration of atropine. Figure 2 shows

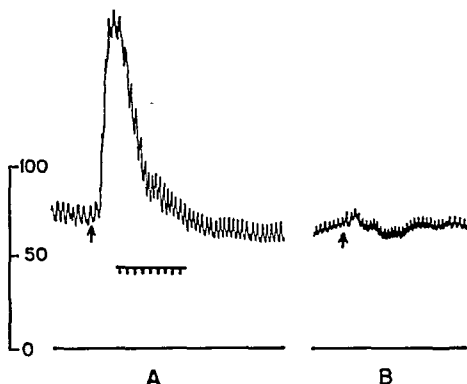


FIG. 2. Carotid blood pressure of spinal cat. Transection C 6-7. Received atropine, 2 mgm./kgm., i.v. A—Chlorimine, 0.01 mM./kgm., i.v., 11:05; Dibenamine HCl, 10 mgm./kgm., i.v., 11:23. B—Chlorimine, 0.01 mM./kgm., i.v., 11:40. Time interval 10 seconds.

a rise in blood pressure following the intravenous injection of 0.01 mM./kgm. of chlorimine in a cat which after a spinal transection at C 6-7 had received 2 mgm./kgm. of atropine. The administration of dibenamine, an adrenergic blocking agent (15), abolished the pressor response. In view of these results and the fact that respiration was not depressed, the vasopressor action can be attributed to stimulation of sympathetic ganglia.

Large doses of chlorimine abolished the circulatory response to vagal stimulation. The agent also elicited transient bradycardia and varying degrees of heart-block as noted in electrocardiographic recordings. The cardiac effects were prevented by prior administration of atropine.

3. *Hydroxyimine*. Intravenous injection of hydroxyimine caused cholinergic effects which resembled those elicited by HN2 or chlorimine. However, larger doses were required for a given response. Doses of 0.02 mM./kgm. were depres-

sor in the absence of atropine but elevated blood pressure in cats previously treated with atropine (figure 3).

Nictitating membrane. Since the pressor effects of HN2, chlorimine, and hydroxyimine indicated stimulation of sympathetic ganglia by these agents, experiments were performed using responses of the cat nictitating membrane to

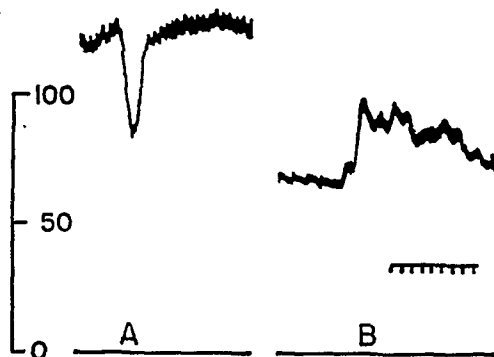


FIG. 3. Carotid blood pressure of cat. A—Hydroxyimine, 0.02 mM./kgm., i.v., 3:23; Atropine sulfate, 2 mgm./kgm., i.v., 3:57. B—Hydroxyimine, 0.02 mM./kgm., i.v., 4:00. Time interval 10 seconds.



FIG. 4. Isotonic recording of nictitating membrane of cat. Received atropine, 1 mgm./kgm. A—Chlorimine, 0.02 mM./kgm., injected into common carotid artery of same side. B—Stimulation of cervical sympathetic trunk 2 minutes after A.

test their actions on the superior cervical ganglion. Figure 4 shows the contraction of the membrane resulting from injection of chlorimine in the common carotid artery. Crushing the superior cervical ganglion abolished this response. No ganglionic blocking effect of chlorimine was evident as judged by equal responses to preganglionic stimulation before and immediately after administration. While the effects of hydroxyimine were similar in this system, it proved to be less potent than chlorimine.

Effects on salivation. Intravenous administration of HN2 or chlorimine in unanesthetized cats caused an immediate salivation of brief duration followed after an interval of 10 to 15 minutes by a second period of copious and prolonged flow. In 1943 Foss and Gaddum (5) noted protracted salivation following intravenous injection of HN2. They also reported the prevention of this response by the prior administration of atropine and the fact that salivation could not be arrested, once initiated, by subsequent administration of atropine.

To investigate this action, further experiments were performed on cats in which the duct of the submaxillary gland was cannulated. Intra-arterial injection

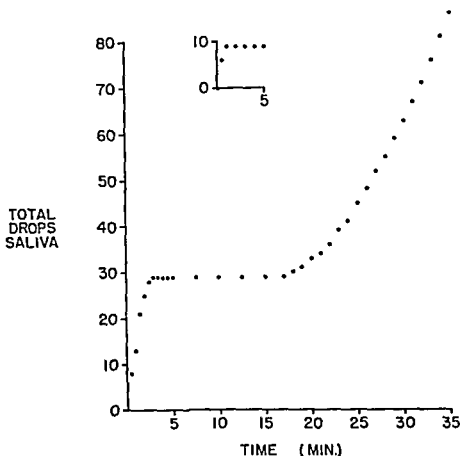


FIG. 5. Salivary flow from right submaxillary gland of cat. Right superior cervical ganglion removed. At 0 time 0.02 mM./kgm. of chlorimine injected into rt. common carotid artery. Note that there was no flow between 3 and 17 minutes after injection. Insert shows typical response to the intra-carotid injection of 10 µgm./kgm. of acetylcholine.

tion of HN2 evoked a unique and discontinuous response (figure 5). An immediate secretion of saliva was followed by a period of about 5 minutes during which there was no flow. At the end of the inactive period salivation was resumed and gradually attained a maximal rate at which it continued for hours. Salivary stimulation was, therefore, separable into two distinct phases the first of which resembled responses to injection of acetylcholine. When atropine was administered prior to HN2 or chlorimine, salivation was completely prevented. However, when atropine (2 mgm./kgm.) was given by intracarotid injection after the initial appearance of salivation, even as early as in the interim between the two phases of salivation, it failed to alter the delayed response.

Although HN2, in high concentrations, has been shown to inhibit choline-

terase *in vitro* (16, 17), this not the mechanism responsible for the protracted salivary stimulation. The submaxillary glands used in these experiments were tested for cholinesterase activity after the administration of HN2 and its transformed products *in vivo*. The mean activity of 17 experimental glands (102.5 cmm. CO₂/30 minutes) did not differ significantly from the controls (108.4 cmm. CO₂/30 minutes). Furthermore, atropine counteracts salivary response to anti-cholinesterase agents irrespective of the order in which it is given in contrast to the fact that it is ineffective when administered after HN2 or its chlorimine.

The unique action of HN2 and chlorimine on the salivary gland suggested the possibility that chemical alterations were involved in the diphasic response. Varying doses of the hydroxyimine were, therefore, injected in an attempt to reduplicate the prolonged salivary flow which followed HN2 or chlorimine. However, the response to hydroxyimine consisted only of a brief burst of saliva-

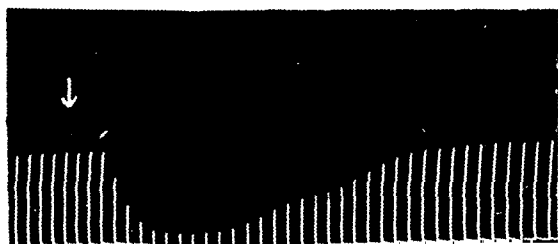


FIG. 6. Isometric recording of cat gastrocnemius-soleus preparation. Stimulation of sciatic nerve with maximal condensor discharges at frequency 1/12 seconds. At arrow intravenous injection of 0.02 mM./kgm. of chlorimine.

tion. Sustained flow was never observed. This response was also prevented by atropine.

Since HN2 and its transformed products caused stimulation of sympathetic ganglia, the possibility remained that at least some of the effects upon the salivary gland might be mediated through this mechanism. However, removal of the ipsilateral superior cervical ganglion did not alter the response of the submaxillary gland to HN2.

Paralytic effects. 1. *Chlorimine.* In order to investigate further the acute paralysis caused by chlorimine, experiments were performed on intact gastrocnemius-soleus preparations of the cat. Figure 6 is an example of the depressed response to maximal motor nerve stimuli which followed 0.02 mM./kgm. intravenously. Rapid recovery ensued and, thereafter, the contractile response remained normal for hours. During the period of severe depression of the response to indirect stimulation, the muscle still responded well to direct stimulation. At the same time the contractile response to close intra-arterial injection of acetylcholine was abolished. Recovery of responses to both acetylcholine

and motor nerve stimulation occurred simultaneously. When chlorimine was injected intraarterially, repetitive, asynchronous contractions could be demonstrated for 1 to 2 minutes before depression of the responses to indirect stimulation ensued.

While the transient depression of contraction can be related to the immediate prostration noted in intact cats receiving chlorimine, there was no effect upon the nerve-muscle preparation which could account for the delayed paralysis which follows the administration of either HN2 or chlorimine in intact animals. The above results are in accord with those of Brown (18) who noted a block in neuromuscular transmission in the frog nerve-sartorius preparation following exposure to HN2 or chlorimine. It is probable that the neuromuscular block which he observed following exposure to HN2 was due to its transformation to chlorimine in the organ bath. In the intact cat HN2·HCl failed to produce an immediate paralysis even following large doses. This may be ascribed to the failure of chlorimine formed *in vivo* from HN2 to attain paralytic concentrations in the circulation.

2. *Hydroxyimine*. In contrast to the immediate effect of chlorimine, even massive doses of hydroxyimine, given intravenously or intra-arterially, caused no depression of responses to maximal single motor nerve shocks or to tetanizing currents (frequency 60/sec.) of maximal intensity. Evans and Foss (6) noted that 24-hour unbuffered aqueous hydrolysates of HN2, which cause a paralytic disorder similar to that produced by hydroxyimine, had no effect on the contraction of the cat quadriceps following stimulation of the femoral nerve when intravenous doses as large as 40 mgm./kgm. had been given. In contrast, 0.5 mgm./kgm. caused definite paralysis in the intact animal. These investigators also found that 24-hour hydrolysates failed to depress spinal reflexes as judged by knee-jerks mechanically recorded in spinal cats. They did, however, note some decrease in the rigidity of decerebrate cats following administration of this agent. While the site and nature of the effect of hydroxyimine remains obscure, the paralysis is clearly not due to an action either at the neuromuscular junction or in the contractile mechanism of muscle.

Intestinal motility. HN2, chlorimine, and hydroxyimine caused stimulation of the isolated rabbit and guinea pig duodenum. The responses were prevented by adequate concentrations of atropine. Similar findings were reported by Foss and Gaddum (5) with rabbit duodenum and afford further evidence of the cholinergic effects of HN2 and its transformations. However, in the intact animal atropine had little effect on the diarrhea resultant from HN2 administration (5).

DISCUSSION. Several groups of investigators have related the biological effects of nitrogen mustards to their known chemical transformations, (7, 8). HN2, as the prototype of this group of compounds may be considered to owe its varied and multiphasic pharmacologic effects to the production of a succession of such transformed products *in vivo*.

The acute pharmacology of HN2 is characterized by the cholinergic actions

of its two cyclic imonium transformation products and by a neurologic disorder produced by the latter of these products. Thus, the effects of the chlorimine of HN2 are manifested by cholinergic effectors of the autonomic nervous system, sympathetic ganglia, and skeletal muscle. In these loci the resultant stimulation or depression is a transient phenomenon, resembling the effects produced by certain choline esters. There is no evidence in the intact animal relating cholinergic actions to inhibition of cholinesterase. As noted by Foss and Gaddum (5) and confirmed in the present study, HN2 does not sensitize the animals to the circulatory effects of acetylcholine. However, Foss and Gaddum did find that doses of HN2, insufficient to stimulate the frog rectus preparation, sensitized the muscle to subsequent application of acetylcholine, an effect which they attributed to an eserine-like action. Nevertheless, the present study failed to reveal an inhibition of cholinesterase in submaxillary tissue of animals which had received doses of either HN2 or chlorimine sufficient to cause prolonged salivation. Furthermore, Feldberg (19) noted that concentrations of HN2 sufficient to reduce cholinesterase activity had an equally depressant effect upon acetylcholine synthesis.

As a cholinergic agent chlorimine is unique in causing a diphasic response of salivary tissue and in the failure of atropine to abolish salivation, once initiated. In contrast to other cholinergic effectors, salivary gland shows a strong affinity for chlorimine which could be conceived to undergo chemical transformation at this site and so evoke the secondary phase of salivary secretion. The latency between completion of the initial secretory response and initiation of the delayed phase might be due to the time required for such transformation to occur *in vivo*. The fact that the second response occurs coincidently with the onset of the delayed neurologic disorder in intact cats suggests this possibility. However, since protracted salivation cannot be duplicated by injection of hydroxyimine, it is difficult to eliminate the alternative possibility that the secondary phase of salivary stimulation represents a delayed response to an interaction with certain receptors which occurs at the time of the initial stimulation. In either case, the fact that prior administration of atropine prevents salivary response to chlorimine, suggests that the effects of the agent can be attributed to interactions with cholinergic receptors. However, it should be noted that Foss and Gaddum (5) attribute the delayed salivary response to a direct action of HN2 on glandular cells not mediated through receptor mechanisms. Obviously the mechanism of action of HN2 or chlorimine in salivary gland is obscure and warrants further study.

The paralytic actions of chlorimine also consist of an initial and a delayed phase. The former is clearly due to an action upon the neuromuscular junction, possibly related to the quarternary onium structure of chlorimine, while the latter is part of a complex neurologic disorder which is central in action. Thus, the two paralytic responses to chlorimine are related to different sites of action. Although the delayed neurologic disorder has not been localized, it may be defined as a disturbance of the motor system manifested by incoordination,

asynergia, progressive muscular weakness with loss of postural tone, and without apparent involvement of the vestibulo-cerebellar system or of the spinal reflex arc. Since animals which survive severe paralysis are normal on the following day, the disorder is probably functional rather than due to anatomic lesions.

The delayed neurological abnormality which follows large doses of HN2 or chlorimine in the cat may be attributed to the production *in vivo* of hydroxyimine for the following reasons: 1, it is manifested by administration of smaller doses of hydroxyimine than of HN2 or chlorimine, 2, a latency exists between administration of HN2·HCl or chlorimine and the production of the syndrome which may be explained by the time required for transformation to occur *in vivo*, and 3, the latent period before onset of the neurological disorder is significantly reduced by administration of hydroxyimine. The greater potency of hydroxyimine in producing the disturbance may be explained by assuming that only a fraction of injected HN2·HCl or chlorimine is transformed to circulating hydroxyimine. The neurological derangement is the most striking action of hydroxyimine and is probably its only significant contribution to the pharmacologic effects of even large amounts of HN2·HCl in the intact animal. It is interesting to note a marked species difference between the cat and rat in susceptibility to this action.

SUMMARY

1. The acute pharmacology of methyl-*bis*(2-chloroethyl)amine (HN2) has been investigated in rats and cats. A standard procedure for transformation of HN2 was adopted which permitted the analysis of the effects of methyl-2-chloroethyl-ethylenimonium (chlorimine), and methyl-2-hydroxyethyl-ethylenimonium (hydroxyimine). The toxicity of these products by intravenous administration has been compared in cats and rats.

2. The chlorimine of HN2 possesses striking cholinergic properties on effectors of the autonomic nervous system, sympathetic ganglia, and striated muscle. It has a unique action upon the salivary gland producing a diphasic response which is prevented by the prior administration of atropine but which is not altered when atropine is given after secretion has begun.

3. The hydroxyimine of HN2 is a less potent cholinergic agent and its principal effect is in the production of a neurologic disorder characterized by ataxia, incoordination, tremors, and muscular weakness.

REFERENCES*

1. GILMAN, A., AND PHILIPS, F. S.: SCIENCE, 103: 409, 1946.
2. "Approaches to tumor chemotherapy," pgs. 285-378. Amer. Assoc. Advan. Science, 1947, Washington, D. C.
3. GOODMAN, L., AND GILMAN, A.: 1942.
4. SMITH, H. W.: 1942.
5. FOSS, G. L., AND GADDUM, J. H.: 1943.
6. EVANS, C. L., AND FOSS, G. L.: 1942.
7. GILMAN, A., GOODMAN, L., AND PHILIPS, F. S.: 1942.

* Where no reference is given, the material has not appeared in the open literature.

8. ANSLOW, W. P. JR., KARNOVSKY, D. A., JAGER, B. V., AND SMITH, H. W.: *THIS JOURNAL*, **91**: 224, 1947.
9. GOLUMBIC, C., FRUTON, J. S., AND BERGMAN, M.: *J. Org. Chem.*, **11**: 518, 1946.
10. AMMON, R.: *Pflüg. Arch. ges. Physiol.*, **233**: 486, 1930.
11. WOLFF, H. G., AND CATTELL, McK.: *Arch. Neurol. and Psychiat.*, **32**: 81, 1934.
12. BOYLAND, E.: *Brit. Jour. Pharmacol.*, **1**: 247, 1946.
13. GOODMAN, L., GILMAN, A., AND PHILIPS, F. S.: 1943.
14. CORDIER, D. G.: 1941.
15. NICKERSON, M., AND GOODMAN, L.: *THIS JOURNAL*, **89**: 167, 1947.
16. THOMPSON, R. H. S.: 1942.
17. BARRON, E. S. G., BARLETT, G. R., AND MILLER, Z. B.: *J. Exper. Med.*, **87**: 489, 1948.
18. BROWN, D. E. S.: 1943.
19. FELDBERG, W.: 1943.

NICOTINE IN BLOOD IN RELATION TO SMOKING¹

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Information concerning the amount of nicotine in the blood of smokers is necessary for a study of the metabolism of this alkaloid in man. Quantitative data are available on nicotine absorbed from cigarette smoke in the respiratory tract (1). The quantity of nicotine excreted in the urine has been determined for smokers (2, 3). Other investigators have reported nicotine levels in milk from lactating women who smoked cigarettes (4, 5). Preliminary observations on nicotine levels in blood during smoking, reported elsewhere (6) have been repeated with a more trustworthy analytical method (7). The new data presented in this paper show that relatively little nicotine is present in the blood even after long periods of heavy smoking.

EXPERIMENTAL. All subjects in this study were healthy adults between the ages of twenty and thirty years, who, except for the slight inhaler group, habitually smoked twenty or more cigarettes daily. In order to keep the analytical "blank" (7) at a minimum value, the subject abstained from fish and from foods or beverages rich in purines for two days preceding the test. The subject smoked freely until late evening of the day preceding the test. On the morning of the test the initial blood sample was drawn by venipuncture before smoking began. During the next seven hours the subject smoked twenty standard brand cigarettes, about two-thirds of each cigarette being smoked during a ten-minute period. Subjects were classified as deep, moderate, and slight inhalers. Cigars were smoked in one experiment and a pipe in another for a comparison with cigarettes.

Nicotine analyses (7). A distillate from alkalinized trichloroacetic acid filtrate of blood was treated with cyanogen bromide-beta naphthylamine reagents for estimating nicotine. The resulting color due to nicotine plus "blank material" was read in the spectrophotometer. A second aliquot of blood filtrate was treated with an activated carbon which selectively removed the nicotine. The distillate from this aliquot gave the amount of "blank material", and the difference between the two values was calculated as nicotine.

The range of nicotine levels in the blood before and after smoking cigarettes is shown for fifteen subjects in table 1. Two striking results are noted. The initial blood sample from each subject contained some nicotine or a nicotine-like substance eight to ten hours after the last cigarette. Secondly, the nicotine level in the blood shows only a slight increase, from 0 to 0.13 mgm./l., over a seven-hour period of heavy smoking. These data cannot be treated by statistical methods because of the small number of subjects in each group and the large overlap of nicotine values. However, there appears to be a significant difference between the deep inhalers and slight inhalers. The former show an aver-

¹ A preliminary report of this study was made before the American Society of Pharmacology and Experimental Therapeutics, Chicago Meeting, May, 1947.

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age initial concentration of 0.20 mgm./l. of nicotine or nicotine-like substances, while the latter average 0.05 mgm./l. The deep inhalers tend to show a larger increase in blood nicotine on smoking than do the slight inhalers.

TABLE 1
Nicotine levels in blood

SUBJECT	NICOTINE IN BLOOD (MG./L.)		
	Before smoking	After smoking	Increase
1 (D)*	0.12	0.25	0.13
2 (D)	0.16	0.18	0.02
3 (D)	0.35	0.43	0.08
4 (D)	0.18	0.26	0.08
5 (M)†	0.16	0.16	0
6 (M)	0.04	0.06	0.02
7 (M)	0.10	0.14	0.04
8 (M)	0.14	0.18	0.04
9 (M)	0.19	0.21	0.02
10 (M)	0.12	0.12	0
11 (M)	0.08	0.13	0.05
12 (M)	0.07	0.08	0.01
13 (S)‡	0.06	0.13	0.07
14 (S)	0.07	0.08	0.01
15 (S)	0.02	0.07	0.05

* D—Deep inhalation.

† M—Moderate inhalation.

‡ S—Slight inhalation.

TABLE 2
Nicotine levels in blood

SUBJECT	NICOTINE IN BLOOD (MG./L.)		
	Before smoking	After smoking	Increase
4*	0.02	0.07	0.05
16*	0.12	0.20	0.08
17†	0.19	0.20	0.01
19†	0	0.02	0.02
20†	0	0.09	0.09
21†	0	0	0
13†	0	0.05	0.05

* Pipe smoked.

† Cigar smoked.

Nicotine levels in blood before and after seven hours of pipe or cigar smoking are shown in table 2. These subjects were cigarette smokers who changed to a pipe or to cigars for the test. Each subject tried to make his smoking with the pipe or cigar equivalent to smoking one pack of cigarettes in the same period. Although strict quantitative comparisons are not justified, it may be noted that

all the nicotine levels in this group fall in the same range found with cigarette smokers. One subject, No. 4, showed a blood nicotine increase of 0.08 mgm./l. on smoking cigarettes and 0.05 on smoking a pipe. Another subject, No. 13, a slight inhaler, showed a rise of 0.07 mgm./l. with cigarettes and 0.05 mgm./l. with cigars. These results suggest that blood nicotine levels show similar rises under the same conditions of smoking by the same subject whether a cigarette, cigar, or pipe is smoked.

In an attempt to identify the material estimated as nicotine, filtrates from blood of smokers were concentrated by distillation and the final solution tested

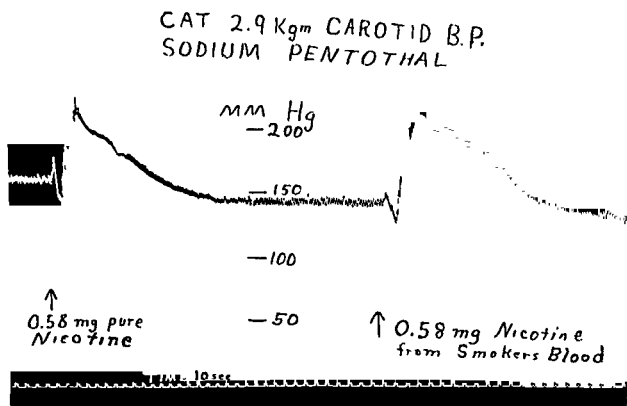


FIG. 1. EFFECT ON BLOOD PRESSURE BY PURE NICOTINE AND BY NICOTINE ISOLATED FROM SMOKERS' BLOOD

for its effect on the blood pressure of a cat.² The final concentrate, which represented nearly three liters of blood, was adjusted to pH 7.3 with hydrochloric acid before injection. The blood pressure tracings, shown in fig. 1, were made with a mercury manometer in the carotid artery of a cat anesthetised with sodium pentothal. With the injection of 0.58 mgm. of pure nicotine in saline solution, the blood pressure showed a small rise, a small drop, and a sudden rise of 80 mm. mercury pressure, then a return to normal in about three minutes. When 0.58 mgm. nicotine isolated from the blood of smokers was injected into the same cat, the blood pressure showed essentially the same changes as with the pure nicotine, an initial rise, a small drop, and a sharp rise of 84 mm. in pressure. This result indicates that nicotine from smokers blood shows both

² Test made by Mr. Russell Barnes.

a qualitative and quantitative effect on blood pressure identical to that shown by pure nicotine.

COMMENTS. All the data presented here suggest a rather efficient mechanism for disposing of nicotine absorbed from tobacco smoke. It may be assumed that some 60 mgm. of nicotine is absorbed from the smoke of twenty cigarettes (1). Were this quantity of alkaloid equally distributed through all water in the body, its concentration would be 1.0 to 1.5 mgm./l. for subjects used in this study. Were the entire dose of nicotine retained in the blood stream, its concentration would exceed 10 mgm./l. The concentration of nicotine actually determined in blood at the end of the smoking period averaged 0.14 mgm./l. for twenty subjects. In view of the fact that nicotine appears to be freely diffusible across body membranes, we accept blood levels as an indication of the quantity of nicotine present in extravascular fluids or tissues. We may conclude that 80 to 95 per cent of the nicotine absorbed from smoke is metabolized during the period of smoking. A similar conclusion has been reached from studies on renal excretion of nicotine by smokers (2, 3).

The presence of nicotine in the initial blood sample drawn at the end of a period of eight or ten hours without smoking suggests that trace amounts of the alkaloid are metabolized or excreted very slowly. In contrast, there appears to be a much more rapid rate of metabolism with the larger doses of nicotine absorbed during smoking.

SUMMARY

Nicotine levels in the blood of smokers have been determined by a chemical method. Subjects who inhale deeply tend to show higher concentrations than do slight inhalers. Nicotine isolated from smokers' blood gave the expected effect on the blood pressure of a cat.

REFERENCES

1. PIERCE, I. H.: *J. Lab. and Clin. Med.*, **26**: 1322, 1941.
2. CORCORAN, A. C., HELMER, O. M., AND PAGE, I. H.: *J. Biol. Chem.*, **129**: 89, 1939.
3. HAAG, H. B., AND LARSON, P. S.: *THIS JOURNAL*, **76**: 235, 1942.
4. THOMPSON, W. B.: *Am. J. Obst. and Gynec.*, **26**: 662, 1933.
5. PERLMAN, H. H., AND DANNENBERG, A. M.: *J. A. M. A.*, **120**: 1003, 1942.
6. WOLFF, W. A., AND HAWKINS, M. A.: *Fed. Proc.*, **6**: 386, 1947.
7. WOLFF, W. A., HAWKINS, M. A., AND GILES, W. E.: *J. Biol. Chem.*, **175**: 825, 1948.

DIATROPINE DERIVATIVES AS PROOF THAT d-TUBOCURARINE IS A BLOCKING MOIETY CONTAINING TWIN ATROPINE-ACETYLCHOLINE PROSTHETIC GROUPS

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Measurements on atomic models of acetylcholine and other drugs with specific muscarinic action have shown that these agents contain two or three oxygen prosthetic groups² at a distance of 5 to 9 Å from one or more methyl on nitrogen prosthetic groups (1). Drugs which block the action of acetylcholine contain in addition to these prosthetic groups blocking or neutralizing moieties such as one or more butyl, or benzyl groups, or a benzohydryl group. Atropine is an extremely potent blocking agent for acetylcholine insofar as muscarinic effects are concerned. Its potency in blocking nicotinic effects of acetylcholine (on autonomic ganglia and on skeletal muscle) is extremely low. In contrast, d-tubocurarine blocks effectively the action of acetylcholine and other nicotinic agents on the skeletal muscle while it exerts little effect on autonomic ganglia, smooth muscle, or glandular cells. In an attempt to correlate spatial relationship of prosthetic groups to drug action, it was of interest to obtain inter-atomic measurements for the active groups of d-tubocurarine.

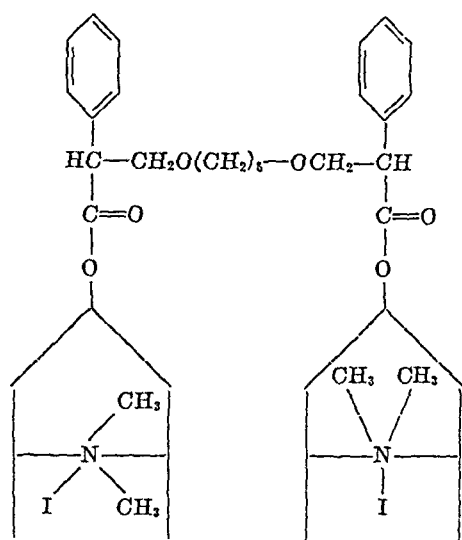
Using Hirschfelder atomic models the distance between the methyl on ring nitrogen and the three oxygen groups on the same hydrogenated quinoline ring of d-tubocurarine is within a radius of 5 to 9 Å (this is in accord with the linear measurements between the prosthetic groups of acetylcholine). The two groups of oxygen atoms in d-tubocurarine average 9 Å in their distance from each other and the nitrogens are 13 to 15 Å apart. d-Tubocurarine, thus, resembles atropine in the "umbrella-structure" and the spatial arrangement of three oxygen groups to methyl on nitrogen. It differs from atropine in that it contains twin rows of these prosthetic groups instead of a single row. Hence, it would appear that the arrangement of twin rows of prosthetic groups is an important factor in endowing the molecule with its specific action on the neuromyal junction.

This hypothesis has been tested by a study of atropine and atropine-like homologues which have been joined together by a chain of approximately 9 Å in length such as is provided by the normal amyl chain (Bovet *et al.* have shown that such amyl diethers have curare action [2]). In making amyl bis molecules of atropine and the quaternary salt of atropine two derivatives have been considered: 1) the dimethiodide of the amyl diether of atropine where two atropine molecules are joined through the tropic acid hydroxyl groups, and 2) the amyl di-quaternary compounds synthesized by connecting two atropine mole-

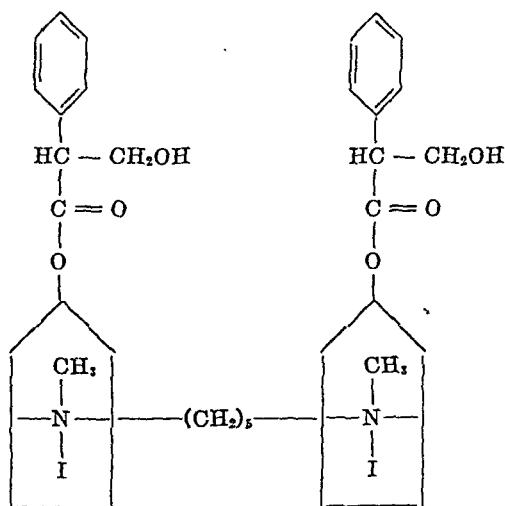
¹ Roche Fellow in Pharmacology.

² In acetylcholine, a methyl on carbon prosthetic group substitutes for the third oxygen prosthetic group while in neostigmine methyl on nitrogen substitutes for the third oxygen.

cules through the tertiary nitrogen with pentamethylene di-iodide (figure 1). Through the unfailing energies of Dr. L. C. Cheney of Bristol Laboratories we



O-Amyl-O-Diatropine Dimethiodide



N-Amyl-N-Diatropine Dimethiodide

FIG. 1. STRUCTURAL FORMULAE OF DIATROPINES

have been provided with these two compounds and similar "alkyl-bis" derivatives of other compounds with atropine-like activity. These are now being studied intensively for their possible practical value. Dibenadryl N-Amyl-N di-iodide was synthesized by Dr. G. Rieveschl, Jr., of Parke, Davis and Company.

The dibenadryl derivative was included because of the known atropine-like action of diphenhydramine (Benadryl) (3). That the findings are distinctly in accord with this hypothesis is shown by the data in table 1.

The compounds were studied for curare-like activity by standardized methods (4). The presence of block at the neuromyal junction was determined by muscular and sciatic nerve faradization in frogs following lymph sac injection. The lethal dose in frogs was

TABLE 1
Relative curare-like effect of atropine and diatropine derivatives

COMPOUND	MOL. WT.	MOUSE I.V.				RABBIT I.V. HEAD DROP	FROG I.L.	
		LD ₅₀	Slope	HD ₅₀	Slope		Periph-eral paral-ysis	Death
Atropine sulfate.	694	95.00 ± 6.2	b = 8.7			60†	1000	1000
Atropine Methyl Nitrate (Eumydrin).	366	11.23 ± 0.89	b = 9.1	5.46 ± 0.44	b = 8.9	8.5	40	>200
Dibenadryl N-Amyl-N Diiodide.	834	4.8 ± 0.24	b = 13			1.25	30	30
Diatropine N-Amyl-N Diiodide.	902	1.23 ± 0.7	b = 12			0.350	30	200
Diatropine O-Amyl-O Dimethiodide.	929	0.79 ± 0.03	b = 19.3	0.58 ± 0.03	b = 12	0.325	7	20
d-Tubocurarine Chloride.	695	0.136 ± 0.003	b = 16.9	0.078 ± 0.002	b = 14.6	0.150	2	>10

* All doses are in mgm./kgm.

† This dose causes head drop followed by lethal convulsions.

determined in order to obtain some measurement of reversibility of the action on the neuromyal junction. The minimum dose causing head-drop (HD) in rabbits was determined by single intravenous infusions over a ninety-second period. Acute toxicity was studied in mice by intravenous injection at a rate of 0.1 cc. per 5 seconds of a suitable concentration of each drug so that a lethal dose was contained in 0.2 to 0.4 cc. The LD₅₀ was calculated according to the method of Litchfield and Fertig (5). Mice succumbing to the various compounds apparently died of respiratory failure, in a manner similar to or identical with that observed with d-tubocurarine chloride. The margin of safety for the mouse was calculated by the ratio: LD₅₀/HD₅₀:

RESULTS. The curare-like action is markedly enhanced by the twinning of

quaternary atropine molecules through an amyl chain. Compared to atropine methylnitrate (eumydrin), both di-atropine compounds are about twenty-five times more effective when tested on rabbits, and about ten times more potent in mice. They show a less marked increase in potency in the frog. d-Tubocurarine chloride, however, is approximately 2 to 2.5 times more effective in rabbits than either di-atropine derivative.

Both di-atropine compounds exhibited a marked degree of specificity in their curarizing effects, as evidenced by the reversibility of their effects in frogs and rabbits. The ratio between curarizing dose (HD_{50}) and lethal dose (LD_{50}) for di-atropine O-amyl-O dimethiodide in mice is 1.36 compared to 1.74 for d-tubocurarine and 2.06 for eumydrin. The effects of the di-atropine compounds in rabbits were of short duration, and the recovery from paralyzing doses was materially aided by neostigmine.

The results furthermore stress the importance of one or more quaternary N atoms for curare-like action of these compounds. The curarizing effects of atropine in intact animals (frogs and rabbits) could only be demonstrated with doses which are convulsively or otherwise fatal to the animals (see table I). In contrast, the curarizing effects of eumydrin and the diatropines are reversible, and death in mammals is directly due to the paralyzing effect on the diaphragm. Furthermore, the non-quaternary diatropine O-amyl-O diether (not listed in the table) has indefinite curare-like action, when compared with diatropine O-amyl-O dimethiodide.

COMMENT. The marked increase in curare-like action by twinning prosthetic groups in a blocking molecule confirms our prosthetic group analysis of the d-tubocurarine molecule. The anatomical implications of this study in regard to the striated muscle cell are extremely interesting. Smooth muscle, glands and heart muscle have the acetylcholine effect blocked by atropine with its single row of prosthetic groups but striated muscle is much more effectively blocked by blocking molecules containing twin rows of prosthetic groups when the mean distance between the oxygen atoms is 9 Å.

The recent studies of Barlow and Ing (6) and others (7, 8) on simple aliphatic di-quaternary diamines indicate that a chain length of C_{10} is optimal for curare-like action of the methylated diamines. These compounds are as potent as d-tubocurarine but are not antidoted by neostigmine. The diamines provide a distance of 15 Å which is in agreement with our measurements on the d-tubocurarine molecule where the distance between the nitrogens is 13 to 15 Å. The ideal arrangement may thus diagrammatically be depicted as in figure 2. Thus the C_{10} chain should be used when the nitrogens are connected. Compounds with slight to moderate atropine action such as diphenhydramine have their toxicity increased and become curare-like when two molecules are connected by an amyl chain. This action should be more markedly enhanced if a C_{10} chain is used to link the two nitrogen atoms. Since the potent aliphatic diamines (6) contain no oxygen prosthetic groups and no blocking moieties (except the aliphatic chain), the careful study of N- C_{10} -N quaternary derivatives will decide between the relative importance of methyl on nitrogen prosthetic groups and the oxygen prosthetic groups.

These studies raise many interesting questions and speculations. Does the neuromyal junction of striated muscle have a front and back door of receptors, both of which must be blocked simultaneously? Does attachment of a single atropine or eumydrin molecule on the striated muscle cell oppose the acceptance of an adjacent atropine molecule? Does this signify that the three oxygen prosthetic groups repel the similar groupings of an adjacent molecule? This might prevent two atropine or eumydrin molecules from occupying adjacent positions on the cell surface except with extremely high dosage when laws of mass action might operate rather than a possible adsorption phenomenon. From a teleo-

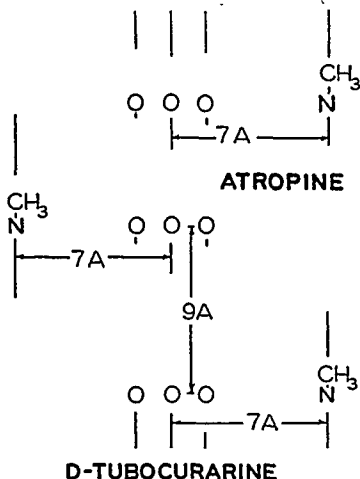


FIG. 2. DIAGRAMMATIC ARRANGEMENT WHICH DEPICTS THE PROSTHETIC GROUPS OF ATROPINE AND D-TUBOCURARINE

The distance between the nitrogens in d-tubocurarine is 13 to 15 A

logical viewpoint the omission of striated (voluntary) muscle from the blocking action of atropine-like compounds may indicate the possible existence (at one time at least) of an atropine-like controlling chemical in the body. For obvious reasons of "fright and flight" the voluntary muscles would of necessity be excepted.

While these studies point to the future synthesis of practical curare substitutes, none of the presently studied compounds have been sufficiently evaluated to suggest their use clinically as substitutes for curare. Due to the widespread action of these compounds on the neuromyal junctions the respiratory depression produced by large doses militates against curare-like compounds having a therapeutic index of more than two when the head drop dose is considered as the effective dose.

SUMMARY

Diatropines, whether synthesized by quaternization through the nitrogen atoms or by connecting the tropic acid hydroxyl groups by an aliphatic chain, have a markedly increased curare-like potency which approaches that of d-tubocurarine. Compounds with slight atropine-like activity become curare-like when two molecules are connected by an alkyl chain of suitable length. When the oxygen prosthetic groups are joined a chain of 9 Å length should be used while a chain of 15 Å length is probably optimal for joining the nitrogen atoms.

REFERENCES

1. PFEIFFER, C. C.: *Science*, **107**: 94, 1948.
2. BOVET, D., COURVOISIER, S., DUCROT, R., AND HORCLOIS, R.: *Compt. rend. Acad. Sci.*, **224**: 1733, 1947.
3. LOEW, E. R., MACMILLAN, R., AND KAISER, M. E.: *THIS JOURNAL*, **86**: 229, 1946.
4. KIMURA, K. K., AND UNNA, K. R.: *Fed. Proc.*, **7**: 233, 1948.
5. LITCHFIELD, J. T., JR., AND FERTIG, J. W.: *Bull. Johns Hopkins Hosp.*, **69**: 276, 1941.
6. BARLOW, R. B., AND ING, H. R.: *Nature*, **161**: 718, 1948.
7. GLOCK, G. E., MOGEY, G. A., AND TREVAN, J. W.: *Nature*, **162**: 113, 1948.
8. PATON, W. D. M., AND ZAIMIS, E. J.: *Nature*, **161**: 718, 1948.

COMPARATIVE PHARMACOLOGY OF THE OPTICAL ISOMERS OF ARTERENOL

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Arterenol, 1-(3,4-dihydroxyphenyl)-2-aminoethanol or noradrenaline, and epinephrine were both synthesized in 1904 by Stolz and Flächer (1), and Dakin (2); a few years later Flächer succeeded in resolving epinephrine into its optical isomers (3). The study of the pharmacological actions of *l*- and *d*-epinephrine and related compounds disclosed the great biological importance of optical isomerism in the field of the sympathomimetic amines, and demonstrated that the *l*-isomers were responsible for most of the sympathomimetic activity of the racemic mixtures. Earlier studies with *d,l*-arterenol revealed that it has a stronger pressor action than *d,l*-epinephrine, and therefore *l*-arterenol was expected to be more active than the known sympathomimetic pressor agents. When one of the theories proposed for neurohumoral transmission of nerve impulses postulated the participation of *l*-arterenol, the importance of the latter was greatly increased. However, the studies on which this theory was based were carried out with the racemic compound since all attempts to separate the optical isomers were unsuccessful. When, in 1948, the resolution of arterenol was accomplished by Tullar (4) the long-sought *l*-isomer became available for pharmacological studies, and particularly for investigations of its role as a neurohumoral agent and of its potential value in therapeutics.

A preliminary report of the separation of the optical isomers of arterenol and a summary of a part of the pharmacologic results discussed here was recently published (5).

EXPERIMENTAL PART. *Blood Pressure.* *d,l*-Arterenol has been reported to be more active than *l*-epinephrine on the pressor response of the chloralosed cat previously sensitized with cocaine and ergotamine (6). A related sympathomimetic amine, Cobefrin (*d,l*-di-hydroxy-nor-ephedrine) has been found by Crismon and Tainter to be more active than both *d,l*-arterenol and *l*-epinephrine in stimulating the heart in the cat heart-lung preparation (7).

Most of the estimations of the relative potency of the sympathomimetic amines have been based on their cardio-vascular effects, and on this function arterenol and not epinephrine is the more active agent. Schultz (8) reported that *d,l*-arterenol exceeded *d,l*-epinephrine in pressor potency in a proportion of 1.5 to 1 on dogs under morphine-ether anesthesia. These results were confirmed by Barger and Dale (9) who found ratios of 1.25-1.5 to 1 and by Raymond-Hamet (10). On urethanized cats Tainter (11) calculated that *d,l*-arterenol was one-third more active than *d,l*-epinephrine. Recently West (12) confirmed this ratio using chloralosed cats.

METHOD. The pressor potencies of isomers of arterenol were determined on dogs anes-

thetized with phenobarbital, 150 mgm. per kgm., intraperitoneally. Carotid blood pressure was recorded by means of a mercury manometer. All injections were made into the femoral vein.

The USP XIII assay procedure for epinephrine was used, modified to the extent that both the standard and the "unknown" drug were given at two levels, the lower being about two-thirds of the higher, and no attempt was made to "match" the responses exactly. The four doses were given in an order determined by a Latin square as suggested by Noel (13) so that the responses to four sets of doses were obtained on each dog. The *l*-arterenol was in the form of the bitartrate monohydrate, the *d*-arterenol was the hydrochloride while the *l*-epinephrine was the USP Reference Standard Epinephrine, which is the base. *l*-Arterenol was assayed using *l*-epinephrine as the standard while *d*-arterenol was assayed against its *l*-isomer. The relative potencies and the weighted average were calculated by the methods

TABLE 1

Relative pressor potencies of l-arterenol, in terms of l-epinephrine, and d-arterenol, in terms of l-arterenol

Each value represents an assay on a separate dog

EXPT. NO.	<i>l</i> ARTERENOL* (<i>l</i> EPINEPHRINE = 100) PER CENT \pm S.E.	EXPT. NO.	<i>d</i> ARTERENOL† (<i>l</i> ARTERENOL = 100) PER CENT \pm S.E.
1	128 \pm 3	7	3.0 \pm 0.2
2	186 \pm 8	8	3.8 \pm 0.4
3	137 \pm 7	9	3.9 \pm 0.3
4	161 \pm 4	10	3.7 \pm 0.2
5	204 \pm 15	11	3.7 \pm 0.3
6	223 \pm 16		
Weighted average.....	158		3.64

* Injected as a solution of its bitartrate monohydrate salt and compared with an equimolecular quantity of USP Reference Standard Epinephrine.

† Injected as a solution of its hydrochloride salt and compared with an equimolecular quantity of *l*-arterenol bitartrate monohydrate.

described by Noel (13) and Miller, Bliss and Braun (14). Table 1 presents the results obtained on two groups of five dogs.

The data in the left-hand column of table 1 indicate that on an equi-molecular basis, *l*-arterenol is approximately 1.58 times as potent a pressor agent as *l*-epinephrine in dogs anesthetized with phenobarbital; on a weight basis, the ratio is 1.70. The standard errors of the individual assays are relatively low indicating a consistency in the respective responses of each dog to the two compounds. However, this consistency is not observed in the data as a whole since the variation from dog to dog is much greater than would be expected from the results on any one dog. The χ^2 test for homogeneity of the individual ratios (14) reveals that such discrepancies would occur through normal sampling much less frequently than once in a thousand times. The variations are a reflection of the qualitative difference between *l*-arterenol and *l*-epinephrine in their effects on the cardio-vascular system of the dog. Because of the heterogeneity, the weighted average of 158 per cent must be regarded with considerable reserva-

tion; the standard error of this average, calculated without regard to the heterogeneity, would be misleading and has been omitted.

In the right-hand half of table 1 similar data on the relative potency of the two optical isomers of arterenol are summarized for five dogs anesthetized with sodium salts of Pentothal and barbital intravenously. As these results indicate, *l*-arterenol is approximately 27 times more active than the *d*-isomer and therefore is responsible for practically all of the pressor activity of the racemic mixture. It will be noted that the variation between dogs in the pressor ratios of the two isomers of arterenol is much less than that observed between the two homologs, *l*-arterenol and *l*-epinephrine.

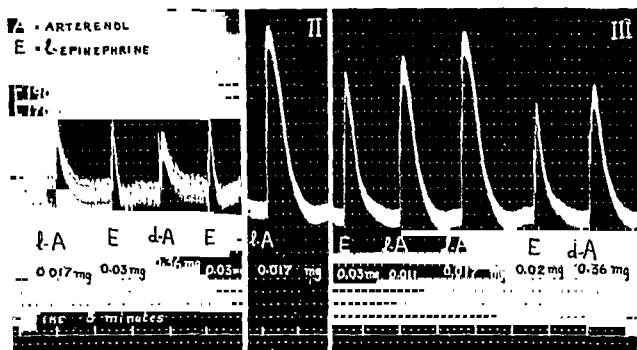


FIG. 1. CAROTID BLOOD PRESSURE TRACINGS FOLLOWING THE INTRAVENOUS INJECTION OF *l*- AND *d*-ARTERENOL (*d*-A AND *l*-A) AND *l*-EPINEPHRINE (E)

Studies were made on an atropinized, 10 kgm. dog anesthetized with 150 mgm./kgm., of phenobarbital intraperitoneally. Between I and II a dose of 8 mgm./kgm. of cocaine was injected subcutaneously.

The pressor responses to equipressor doses of *l*-epinephrine (1–2 microgm. per kgm.) and *l*- and *d*-arterenol differ somewhat in character. In duration, the effect of *l*-epinephrine is the briefest, *l*-arterenol intermediate and *d*-arterenol the longest, the mean ratios for the duration of equivalent pressor doses being in the ratios of 1:1.5:2.1 in dogs. The duration of the pressor effect of *d*-epinephrine has also been found to be longer than that of the levo-form (15, 16). Possibly this difference may be explained by a slower inactivation of *d*-arterenol, due either to the larger dosage or to a lesser affinity of the inactivating enzymatic system for the "unnatural" isomer.

Confirming previous studies with racemic arterenol (Raymond-Hamet, 10, Tainter 11) it was found that the subcutaneous injection of 8 mgm. of cocaine hydrochloride per kgm. sensitized the pressor effects of *l*- and *d*-arterenol. Also in line with earlier observations on racemic arterenol and epinephrine (11, 17), the sensitization to *l*-arterenol was greater than to *l*-epinephrine (fig. 1) or *d*-arterenol.

As an exception among the catecholethylamines, the pressor effect of racemic arterenol is not reversed by ergotoxine (Barger and Dale (9)), yohimbine (Raymond-Hamet (8)) or 933 F (N-piperidino-methyl-benzodioxane) (17). We have found that 2 mgm. per kgm. i.v. of the latter reversed the pressor effect of *l*-epinephrine but only reduced the effect of *l*- and *d*-arterenol.

Action on the heart. A few experiments were carried out on dogs anesthetized with Sodium Pentothal (15 mgm. per kgm.) and sodium barbital (250 mgm. per kgm.) intravenously in which the cardiac activity was recorded with a Jackson myocardiograph attached to the ventricles.

Cardiac acceleration and an increase in the amplitude of the contraction was observed with both *l*- and *d*-arterenol. Doses of *l*-arterenol of 0.3–1.0 microgm. per kgm. i.v., which produced increases in blood pressure of 20–50 mm. Hg, increased the cardiac amplitude from 40 to 300 per cent. The heart rate accelerated slightly with these doses in both normal and atropinized animals.

Equipressor doses of *l*-arterenol, *d*-arterenol and *l*-epinephrine had about the same quantitative effect on the amplitude of contraction (fig. 2). Crismon and Tainter have reported that *d,l*-arterenol is more active than *l*-epinephrine on the heart rate of the cat heart-lung preparation (7).

Perfused rabbit ear. The vasoconstrictor effect of *l*-arterenol was compared to that of *d*-arterenol and *l*-epinephrine on the perfused rabbit ear. The perfusion system was essentially that of Katz (19) as used by Moller to study the vasoconstrictor effect of cocaine (20). The ear artery is attached by a needle and rubber tubing to a vertical glass stand-pipe tube of 100 cm. height and 3 mm. internal diameter, with a funnel-like expansion blown at its upper end. From a Mariotte bottle, the perfusion fluid (Tyrode, at room temperature) falls dropwise into the upper end of the standpipe tube. The flow is regulated by a stopcock to 2.5 to 3.0 cc. per min.; at this rate the pressure is maintained at 25 to 40 cm. of water. As the rate of inflow is constant, any decrease in the caliber of the ear vessels results in a rise in the column of fluid, the magnitude of which is a measure of the vasoconstriction.

The absolute sensitivity and the discriminatory power of the preparation changes gradually during the experiment, increasing the error of quantitative determinations. Doses of 0.04–0.1 microgm. of *l*-epinephrine and 0.05–0.1 microgm. of *d,l*-arterenol injected into the tubing produced marked vasoconstriction. In the eight ears studied *l*-epinephrine was more active than *l*-arterenol with ratios varying from 1.5 to 2.5. On the other hand, *l*-arterenol was found to be from 12 to 18 times more active than the *d*-isomer by this test.

Prolongation of local anesthetic effect of procaine. The capacity of arterenol to extend the duration of local anesthesia was tested on rabbits by injection around the external canthus of the eye. Doses of 0.5 cc. of 0.5 per cent procaine alone and 0.5 per cent procaine with *l*-arterenol in dilutions varying from 1:100,000 up to 1:10,000 were injected and the anesthesia determined by the presence or absence of the corneal reflex.

The results in table 2 show that all the concentrations of arterenol used prolong the local anesthetic effect of procaine. However, a regular curve was not ob-

tained, probably due to the wide individual variations in response. With both *l*-epinephrine and *l*-arterenol a plateau was reached between dilutions of 1:200,000 and 1:50,000. These data appear to confirm previous results obtained by Leser (22) with the same method in rabbits, who found no increase in the duration of anesthesia of procaine above a dilution of *l*-epinephrine of

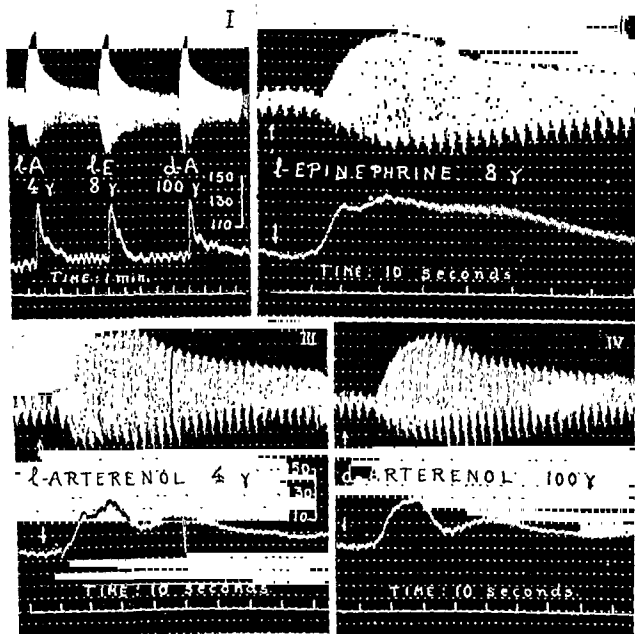


FIG. 2. Dog, 12 KGm. PENTOTHAL-BARBITAL ANESTHESIA

Tracings: Upper, cardiogram (Jackson's myocardiograph). Middle, carotid blood pressure. Lower, time. Intravenous injections.

1:200,000. On the other hand, by the intracutaneous wheal procedure in humans, Bieter (23) found that the optimum dilution of *l*-epinephrine for increasing the local anesthetic effect of 0.125 per cent solution of procaine was 1:200,000 with stronger solutions resulting in a shorter duration of anesthesia. Neither Leser nor Bieter tried *l*-epinephrine 1:10,000, the strongest solution in our tests. The greatest effect was obtained at this concentration with epinephrine and at 1:20,000 with arterenol. At all the concentrations, the effect of epinephrine was more pronounced. As measured by the duration of anesthesia, the vaso-

constriction produced by *l*-epinephrine in the subcutaneous tissue and submucosa around the external canthus of the eye is more prolonged than that produced by equal doses of *l*-arterenol.

Intracutaneous wheal test in guinea pigs. The method of Bülbring and Wajda (24) consists in producing two intracutaneous wheals on the back of each animal by injecting the anesthetic solutions and determining the number of times there is no response to six pinpricks applied in sets to each wheal, every five minutes for 30 minutes. Six animals are used for each dose level. The negative responses obtained in the six readings for each wheal are summed and the mean for the six

TABLE 2

Comparison of l-epinephrine and l-arterenol in prolonging local anesthesia from 0.5% procaine in the external canthus of rabbits

VASOCONSTRICTOR NAME	DILUTION*	pD†	NUMBER OF RABBITS	DURATION IN MIN.	
				Ave.	Range
<i>l</i> -Arterenol	1:1,000,000	6.0	12	36	25- 60
<i>l</i> -Arterenol	1:540,000	5.73	6	50	40- 60
<i>l</i> -Arterenol	1:400,000	5.6	6	72	65- 90
<i>l</i> -Arterenol	1:200,000	5.3	12	79	45-130
<i>l</i> -Arterenol	1:100,000	5.0	10	70	60- 85
<i>l</i> -Arterenol	1:54,000	4.73	5	79	70- 90
<i>l</i> -Arterenol	1:50,000	4.7	10	60	40- 90
<i>l</i> -Arterenol	1:27,000	4.43	6	82	60-115
<i>l</i> -Arterenol	1:10,000	4.0	11	127	85-155
<i>l</i> -Epinephrine	1:500,000	5.7	6	87	60-130
<i>l</i> -Epinephrine	1:200,000	5.3	6	110	90-160
<i>l</i> -Epinephrine	1:100,000	5.0	10	124	65-190
<i>l</i> -Epinephrine	1:50,000	4.2	10	131	95-120
<i>l</i> -Epinephrine	1:25,000	4.4	10	110	70-140
<i>l</i> -Epinephrine	1:10,000	4.0	12	215+	135-300+
Procaine control	0.0		12	17	

* In terms of base.

† Log of reciprocal of the dilution ratio (21).

wheels is calculated. The maximum value of 36 was obtained with all four solutions, thereby indicating complete local anesthesia.

In table 3 the values obtained after 30 minutes represent simply the arithmetic sums of six readings (one for each wheal). These results show the increase in duration of the anesthetic effect of procaine produced by both *l*-epinephrine and *l*-arterenol and at a dilution of 1:200,000 (in terms of the base) they were equally active.

Smooth muscle organs. Retractor penis in situ: The effect of *d,l*-arterenol on the dog's retractor penis has been studied by Barger and Dale (9), who found that it was considerably less active than *d,l*-epinephrine in producing contraction of this muscle. Other primary amines in their series were also found less active than the corresponding methylamines. Cocaine sensitizes the effect of epineph-

rine on the cat's retractor penis (25) and that of epinephrine and other catecholamines on the dog's retractor penis *in situ* (26).

Method. Dogs anesthetized with Sodium Pentothal and sodium barbital were used. In some animals, the sympathetic innervation of the muscle was suppressed by section of the lumbar sympathetic chains. All the drugs were injected into the femoral vein and the resultant changes in carotid blood pressure recorded. After longitudinal section of the skin covering the penis, the anterior part of the muscle was dissected, cut at the site of its anterior insertion and attached by a long thread to a heart lever for recording. Equipressor doses of *l*- and *d*-arterenol produced approximately the same degree of contraction of the retractor penis. Therefore, *l*-arterenol is about 27 times more active than the *d*-isomer on this muscle; however, in comparison with *l*-epinephrine, *l*-arterenol is much less active

TABLE 3

Intracutaneous wheal tests in guinea pigs with 0.1% procaine and epinephrine or arterenol

Method of Bülbring and Wajda (24)

VASOCONSTRICTOR AND DILUTION USED IN TERMS OF THE BASE	MEAN SUM FOR 30 MINUTES	INDIVIDUAL SCORES				
		60 min.	90 min.	120 min.	150 min.	175 min.
<i>l</i> -Epinephrine 1:200,000.	36	34	30	25	20	
<i>l</i> -Epinephrine 1:100,000	36	36	36	36	33	33
<i>l</i> -Arterenol 1:400,000	36	34	26	24	18	17
<i>l</i> -Arterenol 1:200,000.	36	33	28	25	25	23
Procaine 0.1%	5.66 \pm 3.6					
Procaine 0.25%	16.0 \pm 4.2					
Procaine 0.5%	28.0 \pm 2.6					
Procaine 1.0%	31.6 \pm 4.1					

on the retractor penis than on blood pressure. In three dogs, the dose of *l*-arterenol required to induce the same degree of contraction was 4 to 5 times larger than that of *l*-epinephrine. This is illustrated in fig. 3 in which the effect of equiactive doses of *l*-epinephrine and *l*- and *d*-arterenol can be compared.

Isolated rabbit ileum. The optical isomers of arterenol depress the activity of the isolated rabbit ileum. Qualitatively they act like epinephrine. In calculating the potency of *l*- and *d*-arterenol and *l*-epinephrine we have followed the method recently described by Miller, Becker and Tainter (21) for the determination of the activity of spasmolytic drugs, with the difference that we have used the unstimulated ileum. Considered as an all-or-nothing response, a result was recorded as "positive" when the amplitude of the normal contractions was reduced by 75 per cent or more within two minutes. Each dose level was tried on six to 12 strips from a minimum of six rabbits.

The mean values of the ED₅₀ in log dilutions (pD) were: *d*-arterenol: 5.9 \pm 0.07 (= 1:800,000), *l*-arterenol: 7.70 \pm 0.08 (1:50 millions) and *l*-epinephrine: 7.78 \pm 0.12 (1:60 millions). Therefore, *l*-arterenol is about as active as *l*-epinephrine in producing inhibition. In general agreement with the present

results Auman and Youmans (27) found *l*-epinephrine was 1.5 to 2.0 times as active as racemic arterenol and Emilsson (28) and West (12) observed a two-fold difference.

Isolated guinea pig ileum. Both optical isomers of arterenol relax the normal and the histamine-contracted isolated guinea pig ileum. The relative potencies of *d*- and *l*-arterenol and *l*-epinephrine in this respect were determined using at least six strips of the unstimulated ileum for each dose level by the method of Miller, Becker and Tainter.

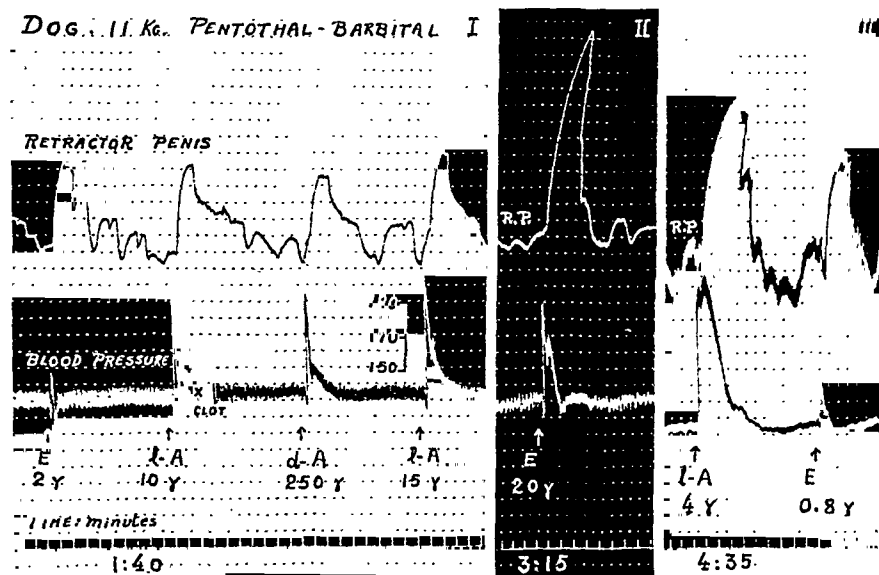


FIG. 3. DOG, 11 KGM. PENTOTHAL-BARBITAL ANESTHESIA

Atropinized. Tracings. Upper: Retractor penis (abdominal sympathetic chains cut); middle: carotid blood pressure; Lower: time, 1 minute intervals. Injections into femoral vein. E = *l*-epinephrine; *l*-A = *l*-arterenol; *d*-A = *d*-arterenol. Total doses in mgm. Between II and III a dose of cocaine HCl was injected subcutaneously.

The guinea pig ileum was found to be more sensitive to these agents than the rabbit ileum. The pD producing positive results in 50 per cent of the strips was $6.58 \pm .09$ (1:3.8 millions) for *d*-arterenol, $8.02 \pm .07$ (1:105 millions) for *l*-arterenol and $8.16 \pm .08$ (1:144 millions) for *l*-epinephrine. Although the standard errors are relatively large, it appears that *l*-epinephrine is slightly more active than *l*-arterenol and that this is 25-30 times more active than the *d*-isomer, a ratio comparable to that obtained on the blood pressure of the barbitalized dog.

Isolated Rabbit Uterus (Non pregnant). The stimulating effects of *l*-arterenol and *l*-epinephrine were compared on the uterine horns of seven rabbits in dilutions of 1:10 million to 1:50 million. The two substances in the concentrations used produced the same degree of stimulation. The effect of *l*-arterenol appears to last longer.

Guinea pig Uterus (Non pregnant). *l*-Epinephrine and *l*-arterenol were tested on the guinea pig uterus, either unstimulated or contracted by adding acetylcholine to give a 1:10 million dilution. The dilutions of the two amines varied from 1:2 million to 1:20 million. *l*-Arterenol was almost ineffective at 1:20 million. The erratic activity of the uteri made the estimation of the potency very difficult; it appears that *l*-epinephrine is from 2 to 10 times more active than *l*-arterenol in producing inhibition of the uterus.¹

NON-PREGNANT RAT UTERUS

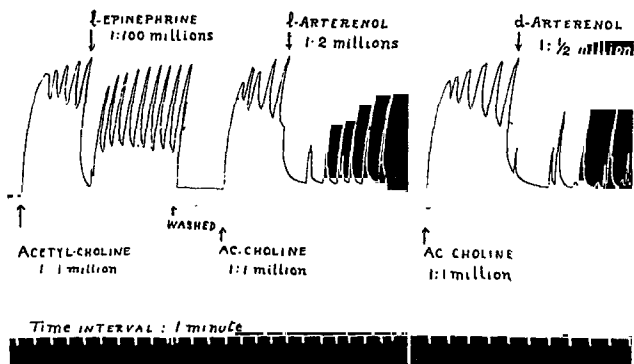


FIG. 4. ISOLATED NON-PREGNANT RAT UTERUS

Rat Uterus (Non pregnant). In the uterus stimulated by acetylcholine (1:1 million to 1:5 million; pD 6-6.7) *l*-arterenol was ineffective at 1:100 million. The threshold dilution seems to be around 1:10 million; a concentration three times this produced a degree of inhibition (decrease in tonus) comparable to that of 1:100 million of *l*-epinephrine. Therefore, *l*-epinephrine is approximately 30 times more active than *l*-arterenol in relaxing the rat uterus stimulated with acetylcholine. However, with equiactive doses, the effect of *l*-arterenol was more prolonged. On the other hand, *l*-arterenol was about four times more active than *d*-arterenol (fig. 4).

¹ In a new series of experiments, carried out in August, 1948, both *l*-arterenol and *l*-epinephrine were found to produce contraction instead of relaxation of non-pregnant guinea pig uterus. Some investigators have obtained both contraction and relaxation of the pregnant as well as the non-pregnant guinea pig uterus, while others have observed either relaxation or contraction. (Literature reviewed by Gruber (44)).

Effect on the Bronchioles. There is an extensive literature on the bronchiolar dilatation produced by the sympathomimetic amines. The effect of *d,l*-arterenol has been studied by Tainter *et al.* (29) on the perfused guinea pig lung. They found that it is only about $\frac{1}{4}$ as active on the average as *l*-epinephrine in relieving the spasms produced by histamine, pilocarpine or barium. On the lung *in situ* (30) with the Jackson method, *d,l*-arterenol is a good bronchodilator against the spasm produced by arecoline. On the perfused guinea pig lung, Siegmund, Granger and Lands (31) found that *l*-epinephrine was at least 10 times more active than *d,l*-arterenol against histamine bronchospasm. They also found *d,l*-arterenol very active on the spasm provoked by histamine inhalation. We have used both the perfused guinea pig lungs by the method of Sollmann and Von Oettingen (32) as modified by Tainter, Pedden and James (29) and the method

TABLE 4

Bronchodilator action of d- and l-arterenol in comparison with l-epinephrine in histamine induced asthma in guinea pigs

DRUGS	DOSE* MG./KGM. I.P.	NO. OF EXPERI- MENTS	AVERAGE TIME†				PER CENT IN- CREASE		DOSAGE RATIO <i>l</i> -EPINEPH- RINE = 1
			Control		Exper.		Onset	Duration	
			Onset	Duration	Onset	Duration			
<i>l</i> -epinephrine.....	0.01	7	0.60	1.04	1.09	1.46	73	35	1
<i>l</i> -epinephrine.....	0.02	7	0.55	0.85	1.30	2.18	136	156	
<i>l</i> -arterenol... ..	0.05	14	0.48	0.84	1.07	1.69	122	101	3
<i>l</i> -arterenol.....	0.1	7	0.50	1.00	1.80	2.70	260	170	
<i>d</i> -arterenol.....	1.0	18	0.60	1.05	1.35	2.05	125	95	60

* All doses expressed in terms of the bases. Drugs were injected intraperitoneally.

† Time expressed in minutes.

devised by Schauman (33) with some modifications as described by Siegmund, Granger and Lands to induce bronchospasm by histamine inhalation.

On the perfused lung, *l*-arterenol in doses of 0.034 to 0.1 mgm. and *d*-arterenol in doses of 3 to 5 mgm. relaxed the bronchioles constricted by histamine (0.02–0.05 mgm.), all the drugs being injected into the perfusion fluid just entering the lung. The *l*-isomer was 50 to 60 times more active than the *d*-isomer, and about $\frac{1}{17}$ as active as *l*-epinephrine.

In the intact guinea pig, *l*- and *d*-arterenol showed antagonism to the spasm induced by inhalation of histamine diphosphate aerosol, as judged by the delays in the onset of initial symptoms and the appearance of asphyxial convulsions (duration). The results have been summarized in table 4.

By this method the ratios differ from those obtained with the perfused lung; *l*-arterenol is approximately 20 times more active than *d*-arterenol and about $\frac{1}{3}$ as active as *l*-epinephrine.

Central Nervous System Stimulation. The comparative activities of *l*-arterenol and *l*-epinephrine in stimulating the central nervous system have been investigated by the rat "jiggle-cage" technique of Tainter and co-workers (34). By

this method the movements of each rat are summated on an electric counter. *l*-Arterenol was administered subcutaneously as the *d*-bitartrate monohydrate salt and *l*-epinephrine as the base dissolved in weak hydrochloric acid solution, but all doses are expressed in terms of the free base for both compounds. A summary of the results is given in table 5.

From the results it may be seen that both *l*-arterenol and *l*-epinephrine produce moderate stimulation in the dose range used. The differences between the two drugs for individual intervals are scarcely beyond the limits of significance, in view of the relatively large standard errors. However, in the first three hours after injection all the hourly means obtained after *l*-epinephrine, with only one exception, are higher than the corresponding means (in time and dosage) for the *l*-arterenol rats. The total increase in revolutions over the control show greater differences inasmuch as the totals include the additional increases after

TABLE 5

Central nervous system stimulation produced in rats by l-epinephrine and l-arterenol as determined by the Jiggle Cage technique of Tainter, et al. (32)

DRUG	DOSE OF BASE MG./ KGM.	NO. RATS	AVERAGE NUMBER OF REVOLUTIONS PER HOUR \pm S.E. AT TIMES SHOWN AFTER DOSING						TOTAL INCREASE IN REVOL. OVER CONTROL
			1 Hr.	2 Hr.	3 Hr.	4 Hr.	5 Hr.	6 Hr.	
Saline	(2 cc.)	22	2.3 \pm 0.5	1.4 \pm 0.2	1.6 \pm 0.3	1.1 \pm 0.3	1.1 \pm 0.2	1.4 \pm 0.3	
<i>l</i> -Epinephrine	0.25	9	7.2 \pm 1.6	10.9 \pm 4.4	3.7 \pm 1.6	2.0 \pm 0.6	1.2 \pm 0.6	1.0 \pm 0.4	17.1
	0.75	12	10.6 \pm 2.4	8.8 \pm 2.3	6.0 \pm 1.3	3.1 \pm 0.9	1.6 \pm 0.6	1.7 \pm 0.5	22.9
	2.0	12	12.0 \pm 1.9	8.0 \pm 1.5	4.6 \pm 0.9	2.3 \pm 0.6	2.6 \pm 0.8	2.8 \pm 0.8	23.4
<i>l</i> -Arterenol	0.25	9	8.9 \pm 2.0	5.0 \pm 0.8	2.2 \pm 0.5	1.2 \pm 0.3	0.7 \pm 0.1	1.4 \pm 0.5	10.9
	0.75	12	9.6 \pm 2.0	5.3 \pm 1.6	1.8 \pm 0.6	0.6 \pm 0.2	0.5 \pm 0.2	0.7 \pm 0.2	11.4
	2.0	12*	7.7 \pm 1.3	4.0 \pm 1.0	1.4 \pm 0.7	1.2 \pm 0.3	0.9 \pm 0.3	0.9 \pm 0.6	7.9

* 1 animal of this group died.

the third hour following the injection. In conclusion it appears that *l*-epinephrine stimulates the central nervous system of the rat approximately twice as much as *l*-arterenol.

DISCUSSION. The results reported here on *l*-arterenol agree, in a general way, with prior knowledge of the pharmacological action of *d,l*-arterenol. As predicted, most of the activity of *d,l*-arterenol is attributable to the *l*-isomer. For arterenol, the average *d:l* pressor potency ratio is 1:27, which is intermediate between the ratios reported for the pairs of close analogues, namely: 1:12 to 1:15 (35, 36) and 1:18.5 (37) for *d*- and *l*-epinephrine and 1:30 (38) for *d*- and *l*-dihydroxynorephedrine. The relative potencies of the two optical isomers observed in various physiological structures or systems are summarized in table 6.

It is somewhat surprising to find that *l*-arterenol is as active as *l*-epinephrine on the small intestine of rabbits. It has been assumed that the primary amines in the catecholethyl- and propylamine series have appreciably less sympathomimetic inhibitor action than the corresponding methylamines.

Examination of table 6 shows that the ratios of *l*-arterenol potency to that of *l*-epinephrine bear no relation to whether an inhibitory or excitatory type of response is involved. *l*-Arterenol is more active on blood pressure and on the dog's heart. It is also more active on the pregnant uterus of cats according to reports in the literature (6, 12, 39). However, with other structures wherein the sympathetic is excitatory (dog's retractor penis and the cat's nictitating membrane (Bacq, 40)), *l*-arterenol is much less active than *l*-epinephrine. Arterenol is also less effective in raising the blood sugar in rabbits as demonstrated by Sahyun (41) with the racemic mixture and by McChesney and McAuliff with the *l*-isomer (42). In addition, *l*-arterenol is as potent as *l*-epinephrine on an inhibitory sympathetic structure (small intestine) while it is much less active on

TABLE 6
Relative potency of l-epinephrine and d- and l-arterenol

TEST OBJECT	<i>l</i> -ARTERENOL RATIO OF EQUIACTIVE DOSES OF <i>l</i> -ARTERE- NOL AND <i>l</i> -EPINEPHRINE	<i>d</i> -ARTERENOL RATIO OF EQUIACTIVE DOSES OF <i>d</i> - AND <i>l</i> -ARTERENOL	TYPE OF RESPONSE
	<i>l</i> -epinephrine = 1	<i>l</i> -arterenol = 1	
Dog, blood pressure.....	0.6	27	E*
Dog, heart in situ.....	0.6	27	E
Dog, retractor penis.....	4-5	27	E
Rabbit, ear perfusion (vasoconstriction).....	1.5-2.5	12-18	E
Rabbit, isolated ileum.....	1	60	I†
Guinea pig, isolated ileum.....	1.5	27	E
Rabbit uterus, nonpregnant.....	1	—	E
Guinea Pig uterus, nonpregnant.....	2-10	—	I
Rat uterus, nonpregnant.....	30	4	I
Guinea Pig lungs			
a) perfusion.....	17	60	I
b) histamine asthma.....	3	20	I

* E—Excitation.

† I—Inhibition.

the two organs in which the sympathetic is excitatory (retractor penis and nictitating membrane). This raises the question whether *l*-arterenol can fulfill the theoretical requirements for an "excitatory" sympathin, as has been suggested by some investigators.

Originally "sympathetic receptive substance" or "myoneural junction" designated a specialized part of the sympathetic peripheral mechanism, through which the effect of the nerve impulses or of epinephrine was mediated, and which depended trophically on the effector cell, since it did not disappear after section and degeneration of the nerve fibers. It was actually a part of the effector cell, but different from the contractile substance upon which the so called "muscuio-tropic" agents were supposed to act. In the newer concepts of neurohumoral transmission, the role played by the receptive substance changed but little; it continued to play the part of the trigger apparatus for the mechanical effect by

becoming the site of action of the ergone or ergones liberated at the periphery by the stimulation of the nerves. As the effect of epinephrine and other closely related compounds on smooth muscle was augmentation or inhibition depending on the type of receptive substances involved (which in turn was revealed by the type of response to the stimulation of the sympathetic nerves) it was assumed that there are two receptive substances, excitatory and inhibitory, which are fundamentally different. This assumption is supported by the fact that during pregnancy when the sympathetic supply of the uterus in the cat and other animals changes from inhibitory to excitatory there is a parallel change in the responses to epinephrine and closely related drugs.

In the case of the organs in which the sympathetic is inhibitory there is no difficulty in accepting the hypothesis of the inhibitory receptive substance, although it should be considered as a working hypothesis until more is known about the mechanism of contraction of the smooth muscle cells. However, the existence of sympathetic inhibitory innervation has been claimed to explain certain inhibitory effects produced by epinephrine and related compounds on the blood vessels, such as the fall of pressure elicited by small doses, the secondary hypotension which follows the normal pressor effect and the reversal obtained after sympatholytic agents. Ergotoxine, 933 F and other sympatholytics should inhibit only the effect of sympathomimetic amines on the excitatory receptive substance, leaving the inhibitory component of the vascular effect unaltered; only those amines which act on both types of receptive substance should depress the blood pressure after sympatholytics. Therefore, according to this theory, arterenol and other nor-compounds have very little or no effect on the inhibitory sympathetic receptive substance. The same theory was advanced to explain the fact that arterenol was more active than epinephrine on the blood pressure while it was less potent on the retractor penis (9). It was assumed that epinephrine was more active than arterenol in producing excitatory effects, as was shown on the retractor penis which has no inhibitory sympathetic supply, while on the blood pressure the pressor effect of epinephrine was reduced by its own inhibitory action, while the effect of arterenol, on the other hand, having a very weak or no inhibitory component, was fully effective. This theory implies the existence of inhibitory sympathetic fibers in every vascular area in which epinephrine acts as a vasodilator after sympatholytics, a generalization for which there is no conclusive evidence, although inhibitory sympathetic fibers have been demonstrated for some vascular areas.

Weight against this hypothesis is provided by the action of compounds such as N-isopropyl-arterenol (Isuprel) and other closely related amines which are predominantly or purely vasodilators in action. Their action and the inhibitory effects of epinephrine are supposed to be similar in nature; that is, on inhibitory sympathetic receptive substances. However, we have made the observation that Isuprel relaxes the atropinized dog's retractor penis *in situ* (fig. 5) and *in vitro*, a muscle which does not have inhibitory sympathetic fibers (43). It also relaxes the atropinized vessels of the perfused ear of the rabbit, an animal in which ergotoxine does not reverse the pressor effect of epinephrine. Apparently

the site of action of Isuprel on smooth muscle cells is not identified anatomically or physiologically with inhibitory sympathetic fibers and nerve endings. The site of the inhibitory action of epinephrine is presumably the same, considering the close chemical and pharmacological relationship of the two compounds.

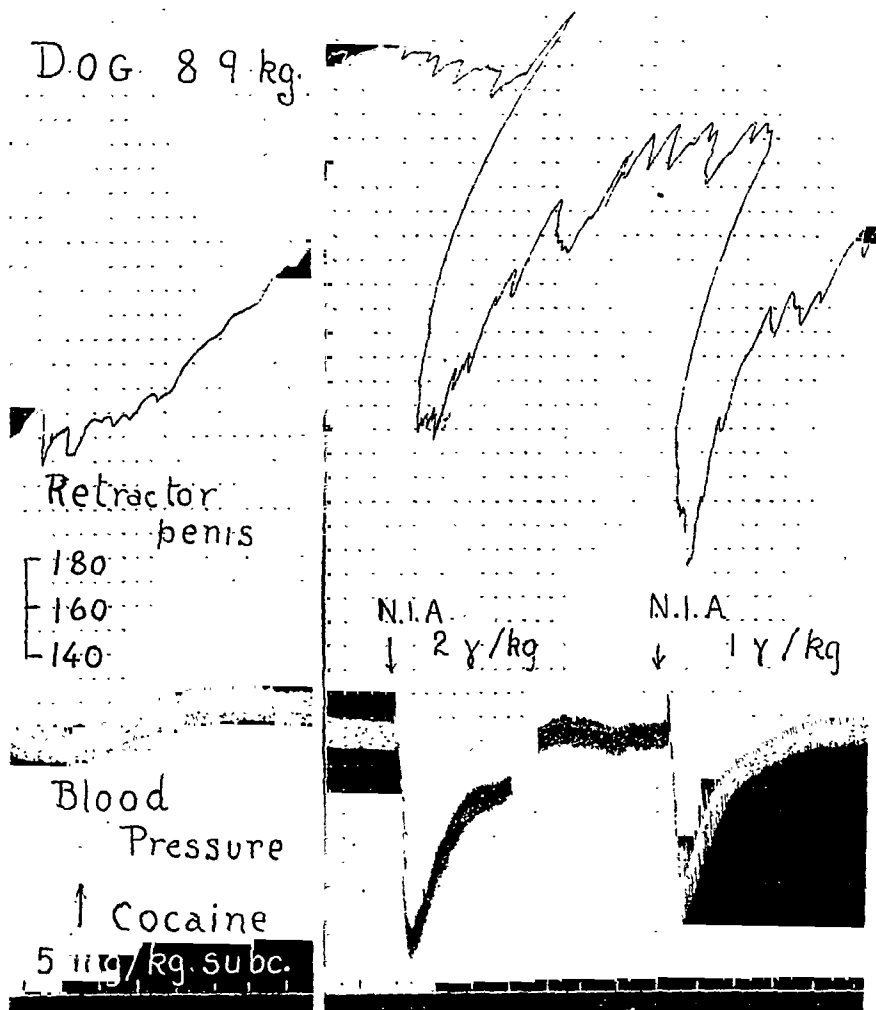


FIG. 5. Dog, 8.9 KGM. PENTOTHAL-BARBITAL ANESTHESIA

Tracings: Upper: retractor penis. Middle: carotid blood pressure. Lower: time in minutes. Intravenous injections. The tracings show the augmentor effect of cocaine and the inhibition produced by N-isopropyl-arterenol (Isuprel).

The fact that arterenol, epinephrine and Isuprel produce a combination of augmentation and inhibition of effector cells implies to us that in the chemical structure of the group of compounds known as sympathomimetic, the possibility exists of influencing in opposite directions the same biochemical mechanism which in the smooth muscle cell is responsible for or directly involved in the

mechanical responses. The affinity for the same cellular mechanism would depend on the structural features which they have in common while the type of amine group would be responsible for the direction and degree in which this mechanism is influenced.

CONCLUSIONS

1. The pharmacologic actions of *l*- and *d*-arterenol have been compared with *l*-epinephrine on the blood pressure, cardiac activity and the retractor penis of the barbitalized dog, on the isolated ileum of the rabbit and guinea pig, and the bronchioles of guinea pigs. The effects of *l*-arterenol were also compared to that of *l*-epinephrine on the uterus of rabbits and guinea pig, on the duration of the local anesthesia from procaine, and on the spontaneous random activity of the rat by the jiggle-cage technique.

2. On the barbitalized dog *l*-arterenol is 1.70 more active than *l*-epinephrine in raising blood pressure, while *d*-arterenol is about $\frac{1}{2}$ th as active as the *l*-isomer. Equipressor doses of these drugs produce about the same degree of cardiac stimulation as judged by the increase in heart rate and amplitude.

3. On the dog's retractor penis *in situ*, equipressor doses of *l*- and *d*-arterenol have the same stimulating effect. *l*-Epinephrine is 4 to 5 times more active than *l*-arterenol.

4. Cocaine sensitizes the effect of *l*- and *d*-arterenol on the blood pressure and the retractor penis.

5. On the isolated rabbit ileum *l*-arterenol is approximately as active as *l*-epinephrine in producing inhibition: *d*-arterenol is about $\frac{1}{4}$ th as active. On the isolated guinea pig ileum *l*-arterenol is somewhat less active than *l*-epinephrine in inducing relaxation while it is 20 times more active than *d*-arterenol.

6. The isolated rat uterus stimulated with acetylcholine is promptly relaxed by *l*- and *d*-arterenol. On this preparation, *l*-arterenol is about 4 times more active than the *d*-isomer and $\frac{1}{3}$ th as active as *l*-epinephrine. In stimulating the isolated rabbit uterus *l*-arterenol and *l*-epinephrine are equally active, while the latter is 2 to 10 times more active than the former in inducing inhibition of the isolated nonpregnant guinea pig uterus.

7. In the perfused guinea pig lung *l*-epinephrine is about 17 times more active than *l*-arterenol which is 50 to 60 times more active than *d*-arterenol as a bronchodilator against histamine constriction. Against the bronchospasm induced by nebulized histamine in the intact guinea pig *l*-epinephrine is about 3 times more active than *l*-arterenol which is 20 times more active than *d*-arterenol.

8. *l*-Epinephrine is more potent than *l*-arterenol in increasing the duration of the local anesthetic effect of procaine.

9. Both *l*-epinephrine and *l*-arterenol moderately increase the spontaneous activity of the rat as judged by the jiggle cage method. The degree of stimulation is greater with *l*-epinephrine.

10. These results are not compatible, in any simple way, with certain current theories that identify *l*-arterenol as being the augmentor hormone of the sympathetic nervous system.

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REFERENCES

1. STOLZ, F., AND FLÄCHER, F.: D.R.P. 157,300 (Frdl., 7: 689); U. S. Patents No. 862,675; 930,703.
2. DAKIN, H. D.: J. Physiol., 32: 34, 1905. Proc. Roy. Soc., Ser. B. 76: 491, 498, 1905.
3. FLÄCHER, F.: Ztschr. f. physiol. Chem., 58: 189, 1908.
4. TULLAR, B. F.: J. Am. Chem. Soc., 70: 2067, 1948.
5. TAINTER, M. L., TULLAR, B. F., AND LUDUENA, F. P.: Science, 107: 39, 1948.
6. VON EULER, U. S.: Acta Physiol. Scand., 12: 73, 1946.
7. CRISMON, J. M., AND TAINTER, M. L.: THIS JOURNAL, 64: 190, 1938.
8. SCHULTZ, W. H.: Bull. 55, Hygienic Lab. U. S. Public Health, 1909.
9. BARGER, G., AND DALE, H. H.: J. Physiol., 41: 19, 1910.
10. RAYMOND-HAMET: Compt. rend. Acad. Sci., 191: 869, 1930.
11. TAINTER, M. L.: Arch. internat. pharmacodyn. et de therap., 41: 365, 1931.
12. WEST, G. B.: J. Physiol., 106: 418, 1947.
13. NOEL, R. H.: THIS JOURNAL, 84: 278, 1945.
14. MILLER, L. C., BLISS, C. I., AND BRAUN, H. A.: J. Am. Pharm. A., 28: 644, 1939.
15. FROHLICH: 1909, cit. by SOLLMANN, T.: Pharmacology, Sixth Edition, p. 422.
16. TAINTER, M. L.: THIS JOURNAL, 40: 43, 1930.
17. MELVILLE, K. I.: THIS JOURNAL, 59: 317, 1937.
18. RAYMOND-HAMET: Compt. rend. Soc. de Biol., 97: 618, 1927.
19. KATZ, G.: Arch. internat. pharmacodyn. et de therap., 49: 239, 1934.
20. MOLLER, K.: Arch. internat. pharmacodyn. et de therap., 57: 51, 1937.
21. MILLER, L. C., BECKER, T. J., AND TAINTER, M. L.: THIS JOURNAL, 92: 260, 1948.
22. LESER, A. J.: Anesthesiol., 1: 205, 1940.
23. BIETER, R. N.: Am. J. Surg., 34: 500, 1936.
24. BÜLBRING, E., AND WAJDA, I.: THIS JOURNAL, 85: 78, 1945.
25. OPPENHEIMER, M. J.: Am. J. Physiol., 122: 745, 1938.
26. LUDUENA, F. P.: Rev. Soc. Arg. Biol., 14: 535, 1938.
27. AUMAN, K. W., AND YOUNG, W. B.: Proc. Soc. Exp. Biol. and Med., 42: 111, 1939.
28. EMILSSON, B.: Acta Physiol. Scand., 3: 335, 1941.
29. TAINTER, M. L., PEDDEN, J. R., AND JAMES, M.: THIS JOURNAL, 51: 371, 1934.
30. CAMERON, W. M., AND TAINTER, M. L.: THIS JOURNAL, 57: 152, 1936.
31. SIEGMUND, O. H., GRANGER, H. R., AND LANDS, A. M.: THIS JOURNAL, 90: 254, 1947.
32. SOLLMANN, T., AND VON OETTINGEN, W. F.: Proc. Soc. Exp. Biol. and Med., 25: 692, 1928.
33. SCHAUMANN, O.: Arch. f. exp. Path. u. Pharmacol., 196: 109, 1940.
34. SCHULTE, J. N., REIF, E. C., BACHER, J. A., JR., LAWRENCE, W. S., AND TAINTER, M. L.: THIS JOURNAL, 71: 62, 1941.
35. CUSHNY, A. R.: J. Physiol., 38: 259, 1909.
36. ABDERHALDEN, E., AND MÜLLER, F.: Ztschr. f. Physiol. Chem., 58: 185, 1908.
37. SWANSON, E. E., SCOTT, C. C., LEE, H. M., AND CHEN, K. K.: THIS JOURNAL, 78: 329, 1943.
38. TIFFENEAU, M.: Congress de Physiol. Paris, 1920.
39. GREER, C. M., PINKSTON, J. D., BAXTER, J. H., JR., AND BRANNON, E. S.: THIS JOURNAL, 62: 189, 1938.
40. BACQ, Z. M.: Arch. internat. Physiol., 40: 297, 1935.
41. SAHYUN, M.: Arch. internat. pharmacodyn. et de therap., 45: 285, 1933.
42. MCCHESENEY, E. W., AND McAULIFF, J. P.: unpublished results.
43. LUDUENA, F. P.: unpublished results.
44. GRUBER, G. M.: Physiol. Rev., 13: 55, 1933.

EFFECTS OF TETRAETHYLAMMONIUM BROMIDE ON THE PARASYMPATHETIC NEUROEFFECTOR SYSTEM¹

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Acheson and Pereira (1), in 1946, demonstrated that tetraethylammonium bromide blocks the synapse of sympathetic ganglia. In the same year Acheson and Moe (2) interpreted some of the actions of this drug on the frequency of the heart beats as due to depression of the parasympathetic ganglion formations of the heart.

It has been considered of interest to establish the possibility of blocking the ganglionic synapse of the parasympathetic system with the drug and also to study the effect of the drug on the transmission between the cholinergic postganglionic fiber and the effector, since adrenergic transmission is unhampered by the drug (1).

METHODS. Cats anesthetized with an intraperitoneal injection of sodium pentobarbital-urethane were used (sodium pentobarbital 0.03 grams to 1 cc. of 25 per cent urethane; 1 cc. per kilogram of animal weight). A tracheal cannula was employed so that artificial respiration could be performed when necessary.

The structures used were the superior cervical ganglion (sympathetic) and the ciliary ganglion (parasympathetic).

To stimulate parasympathetic preganglionic and postganglionic fibers, the method of Lucio and Salvestrini (3) was employed. The preganglionic and postganglionic sympathetic fibers were stimulated in the cervical region.

The nerves were stimulated by short rectangular pulses of 0.0005 sec. duration, the frequency of which was controlled by electronic valves. Maximal stimulus was used in the majority of the experiments, and submaximal in the rest.

The drug chosen—tetraethylammonium bromide (Kodak)—was administered intravenously (via the femoral vein). The action of the drug was recorded by photographing the variations in pupillary diameter at different times during stimulation.

A total of 29 experiments was performed. In nearly all the animals the adrenal glands were removed.

RESULTS. A) *Preganglionic parasympathetic fibers.* The intravenous injection of tetraethylammonium bromide during stimulation of the third pair (preganglionic fibers of the ciliary ganglion) at frequencies that fluctuated between 30 and 60 per second and with maximal intensity produced a reduction or abolition of the pupillary reaction. The effect can be observed 1 minute after the administration of the drug and lasts for a full 30 minutes.

In nearly all the experiments the preganglionic sympathetic fibers were stimulated simultaneously on opposite sides; it was possible to observe a blocking of both pupillary reactions with doses fluctuating between 0.06 and 30 mgm. per kilogram of body weight. In one (fig. 1), progressively larger doses were ad-

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REFERENCES

1. STOLZ, F., AND FLÄCHER, F.: D.R.P. 157,300 (Frdl., 7: 689); U. S. Patents No. 862,675; 930,703.
2. DAKIN, H. D.: *J. Physiol.*, **32**: 34, 1905. *Proc. Roy. Soc., Ser. B.* **76**: 491, 498, 1905.
3. FLÄCHER, F.: *Ztschr. f. physiol. Chem.*, **58**: 189, 1908.
4. TULLAR, B. F.: *J. Am. Chem. Soc.*, **70**: 2067, 1948.
5. TAINTER, M. L., TULLAR, B. F., AND LUDUENA, F. P.: *Science*, **107**: 39, 1948.
6. VON EULER, U. S.: *Acta Physiol. Scand.*, **12**: 73, 1946.
7. CRISMON, J. M., AND TAINTER, M. L.: *THIS JOURNAL*, **64**: 190, 1938.
8. SCHULTZ, W. H.: *Bull. 55, Hygienic Lab. U. S. Public Health*, 1909.
9. BARGER, G., AND DALE, H. H.: *J. Physiol.*, **41**: 19, 1910.
10. RAYMOND-HAMET: *Compt. rend. Acad. Sci.*, **191**: 869, 1930.
11. TAINTER, M. L.: *Arch. internat. pharmacodyn. et de therap.*, **41**: 365, 1931.
12. WEST, G. B.: *J. Physiol.*, **106**: 418, 1947.
13. NOEL, R. H.: *THIS JOURNAL*, **84**: 278, 1945.
14. MILLER, L. C., BLISS, C. I., AND BRAUN, H. A.: *J. Am. Pharm. A.*, **28**: 644, 1939.
15. FROHLICH: 1909, cit. by SOLLMANN, T.: *Pharmacology*, Sixth Edition, p. 422.
16. TAINTER, M. L.: *THIS JOURNAL*, **40**: 43, 1930.
17. MELVILLE, K. I.: *THIS JOURNAL*, **59**: 317, 1937.
18. RAYMOND-HAMET: *Compt. rend. Soc. de Biol.*, **97**: 618, 1927.
19. KATZ, G.: *Arch. internat. pharmacodyn. et de therap.*, **49**: 239, 1934.
20. MOLLER, K.: *Arch. internat. pharmacodyn. et de therap.*, **57**: 51, 1937.
21. MILLER, L. C., BECKER, T. J., AND TAINTER, M. L.: *THIS JOURNAL*, **92**: 260, 1948.
22. LESER, A. J.: *Anesthesiol.*, **1**: 205, 1940.
23. BIETER, R. N.: *Am. J. Surg.*, **34**: 500, 1936.
24. BÜLBRING, E., AND WAJDA, I.: *THIS JOURNAL*, **85**: 78, 1945.
25. OPPENHEIMER, M. J.: *Am. J. Physiol.*, **122**: 745, 1938.
26. LUDUENA, F. P.: *Rev. Soc. Arg. Biol.*, **14**: 535, 1938.
27. AUMAN, K. W., AND YOUNG, W. B.: *Proc. Soc. Exp. Biol. and Med.*, **42**: 111, 1939.
28. EMILSSON, B.: *Acta Physiol. Scand.*, **3**: 335, 1941.
29. TAINTER, M. L., PEDDEN, J. R., AND JAMES, M.: *THIS JOURNAL*, **51**: 371, 1934.
30. CAMERON, W. M., AND TAINTER, M. L.: *THIS JOURNAL*, **57**: 152, 1936.
31. SIEGMUND, O. H., GRANGER, H. R., AND LANDS, A. M.: *THIS JOURNAL*, **90**: 254, 1947.
32. SOLLMANN, T., AND VON OETTINGEN, W. F.: *Proc. Soc. Exp. Biol. and Med.*, **25**: 692, 1928.
33. SCHAUMANN, O.: *Arch. f. exp. Path. u. Pharmacol.*, **196**: 109, 1940.
34. SCHULTE, J. N., REIF, E. C., BACHER, J. A., JR., LAWRENCE, W. S., AND TAINTER, M. L.: *THIS JOURNAL*, **71**: 62, 1941.
35. CUSHNY, A. R.: *J. Physiol.*, **38**: 259, 1909.
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37. SWANSON, E. E., SCOTT, C. C., LEE, H. M., AND CHEN, K. K.: *THIS JOURNAL*, **78**: 329, 1943.
38. TIFFENEAU, M.: *Congress de Physiol. Paris*, 1920.
39. GREER, C. M., PINESTON, J. D., BAXTER, J. H., JR., AND BRANNON, E. S.: *THIS JOURNAL*, **62**: 189, 1938.
40. BACQ, Z. M.: *Arch. internat. Physiol.*, **40**: 297, 1935.
41. SAHYUN, M.: *Arch. internat. pharmacodyn. et de therap.*, **45**: 285, 1933.
42. MCCLESNEY, E. W., AND McAULIFF, J. P.: unpublished results.
43. LUDUENA, F. P.: unpublished results.
44. GRUBER, G. M.: *Physiol. Rev.*, **13**: 55, 1933.

graph) and after stimulation (bottom one) are due to the absence of the light reflex by the blocking effect of the drug on the ciliary ganglion. An intense effect on the sympathetic ganglion appears after the first injection at B and the complete blocking effect after the second one at C, as proved by the fact that a double dose administered at D does not modify the pupillary diameter and by the fact that it is equal to the diameter after stimulation. In the parasympathetic ganglion the effect is observed only after the second injection, and it is not yet complete after the third one; i.e., the pupillary diameter is smaller than the one before or after stimulation.

In other words, the effect on the parasympathetic ganglion was obtained in this experiment with doses about four times larger than the one required to block the sympathetic ganglion.

In other experiments the ciliary ganglion was acutely removed on the same side as the sympathetic stimulation, and the blocking effects were of the same magnitude as the experiment of fig. 1.



FIG. 2. DEPRESSOR AND POTENTIATION EFFECTS WITH SMALL AND LARGE DOSES DURING STIMULATION OF THE POSTGANGLIONIC PARASYMPATHETIC FIBERS WITH MAXIMAL STIMULI

A and E show the beginning and ending of stimulation. At B, C and D, tetrathylammonium bromide: 3 mgm., 8 mgm. and 160 mgm. per kilogram of body weight, respectively

B) *Postganglionic parasympathetic fibers.* During stimulation of the postganglionic parasympathetic (cholinergic) fibers at a frequency of 25 to 50 per second, various effects can be observed according to the dose used: With 10 to 100 mgm. per kilogram of body weight, block of the response of the effector is observed similar to the autonomic-ganglion effect.

With doses between 200 and 300 mgm. per kilogram of body weight, it is often observed that an increase in the pupillary contraction takes place. After this potentiation of the response, which lasts nearly 1 minute, a depression and blocking is observed, as shown by a prolonged pupillary dilatation.

The depression with small doses and potentiation with large ones has been observed with both maximal and submaximal stimuli (figs. 2 and 3). The depressor effect can be diminished if the intensity of the stimulus is increased, regardless of whether maximal or submaximal stimulation is employed, but the increase was never greater than that obtained with a maximal stimulus.

In 2 experiments it was observed, as reported by Acheson and Pereira (1), that the response of smooth muscle to acetylcholine (in this case the pupillary sphincter) is not altered by the previous administration of the drug (fig. 4).

C) *Direct effect on the pupil.* It was considered interesting to know the effect produced by direct action of the drug on the pupil. Four cats were used. In one the sympathetic and ciliary ganglia of both sides were acutely removed, and in the other three the superior cervical ganglion of one side was excised 5 to 7 days previously and the rest of the ganglia acutely removed. Under these conditions the drug produces a slight increase of the pupillary diameter (fig. 5)—a

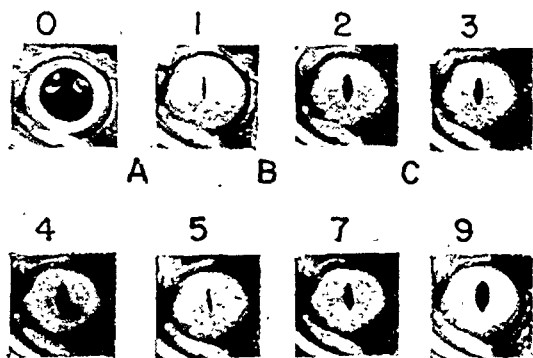


FIG. 3. POTENTIATION WITH LARGE DOSES DURING SUBMAXIMAL STIMULATION OF THE POSTGANGLIONIC PARASYMPATHETIC FIBERS

At A, stimulation begins, with maximal stimuli. At B, intensity is reduced (submaximal). At C, injection of tetraethylammonium bromide: 160 mgm. per kilogram of body weight.

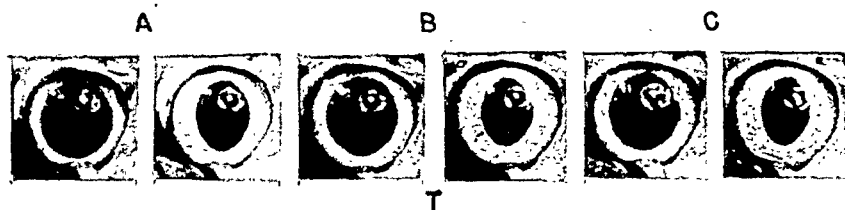


FIG. 4. EFFECT OF THE DRUG ON ACETYLCHOLINIC RESPONSE OF THE PUPIL

Chronic sympathetic postganglionic denervation (6 days) and acute decentralization (removal of ciliary ganglion). At A, B and C, injections of 50 micrograms of acetylcholine. T shows intravenous injection of 16 mgm. per kilogram of body weight of tetraethylammonium bromide, immediately before B.

reaction that is, of course, more intense when the initial tension of the pupil is greater.

In the different experiments, it was never observed that the drug produced pupillary contraction *per se*, even with doses as large as 300 mgm. per kilogram. From this, one may infer that the drug has no muscarinic effect, confirming previous reports, such as those cited by Acheson and Moe (2).

DISCUSSION. Luco and Mesa (4) demonstrated that curare produces a blocking of the synapse in which acetylcholine is mediator. Tetraethylammonium

bromide has a similar action on the neuroeffectors studied; thus Acheson and Pereira (1) observed a depressor effect on the sympathetic ganglion, and this paper studies its action on parasympathetic ganglia and on the postganglionic cholinergic effector system. The observation of Acheson and Pereira that, like curare, it does not block the postganglionic adrenergic effector system is confirmed.

The dilatation of the pupil observed with the administration of the drug, while stimulating the ciliary fibers, is not due to a direct effect of tetraethylammonium bromide on the pupillary sphincter, since during postganglionic sympathetic stimulation there is no effect of the drug on the dilator of the pupil. On the other hand, the magnitude of direct effect of the drug on the pupil dilatation is small

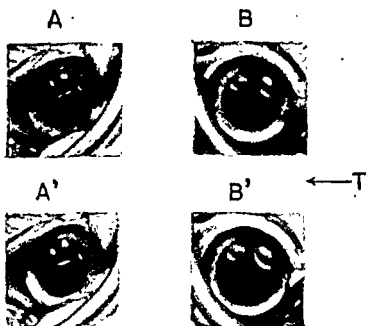


FIG. 5. DIRECT EFFECT OF THE DRUG ON THE PUPIL

A and A' (right side) show the acutely decentralized pupil. B and B' (left side), the acutely decentralized and chronically denervated pupil (removal of the superior cervical ganglion). At T, injection of tetraethylammonium bromide: 25 mgm. per kilogram of body weight.

whereas the depression during postganglionic stimulation may be complete. These considerations therefore exclude the possibility that the depressor effect is due principally to a change in the smooth-muscle contractile system.

It does not at present seem likely that the drug has an effect on the postganglionic axon, since no depression of the postganglionic sympathetic fibers is observed.

It is not possible to accept an increase in the threshold to acetylcholine, which would easily explain the depressor effect, since both in the experiments of Acheson and Pereira (1) and in these, the threshold of the nictitating membrane and the pupil to acetylcholine remained unaltered.

The possibility of a change in the liberation of the mediator would explain the results obtained, but this interpretation has not been submitted to experimental proof.

The transitory, but constant, potentiation produced by the drug in high doses during stimulation is not due to direct action on the muscle, since such a reaction has never been observed, as pointed out in Section C above. This increase in the contraction could also be explained as due to an alteration in the liberation of the mediator.

SUMMARY

The effect of tetraethylammonium bromide on the parasympathetic ganglion and the postganglionic cholinergic effector system was studied on cats anesthetized with sodium pentobarbital.

During stimulation of the preganglionic fibers of the ciliary ganglion, a blocking of the transmission of the impulse through the ganglion was observed.

The stimulation of the postganglionic fibers revealed a blocking of the pupillary response with intravenous doses of 10 to 100 mgm. per kilogram of body weight, and a potentiation of the response with doses from two to three times larger.

REFERENCES

- (1) ACHESON, G. A., AND PEREIRA, S. A.: *THIS JOURNAL*, **87**: 273, 1946.
- (2) ACHESON, G. A., AND MOE, G. K.: *THIS JOURNAL*, **87**: 220, 1946.
- (3) LUCO, J. V., AND SALVESTRINI, H.: *J. Neurophysiol.*, **5**: 27, 1942.
- (4) LUCO, J. V., AND MESA, J.: *Ciencia (Méjico)*, **2**: 298, 1941.

THE ADRENERGIC BLOCKING EFFECT OF CERTAIN β -CHLOROETHYL AMINES¹

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Many compounds, both natural and synthetic, have been found to possess adrenergic blocking activity. The clinical application of such agents has been limited by their toxicity and brief duration of action. In 1946 Nickerson and Goodman (1) reported that dibenzyl β -chloroethyl amine (dibenamine) was capable of blocking certain adrenergic responses for a period up to several days after a single dose. Subsequently Loew, Kaiser, and Anderson (2) described the similar effects of other β -halogenated ethyl amines, namely: benzhydrylethyl β -chloroethyl amine, β -biphenoxyethyl β -chloroethyl amine, and α -naphthyl-methyl ethyl β -chloroethyl amine.

This group of compounds is presumed to undergo intramolecular cyclization under physiological conditions with the formation of a highly reactive imine ring. It is to this transformed state of the dibenamine molecule that the adrenergic blocking effect has been ascribed (3). This effect can be prevented by the prior administration of thiosulfate, an action which is attributed to the ability of this ion to combine with the imine form of such compounds with the production of an inactive ethyl thiosulfate derivative.

The present study is concerned with the relationship between chemical structure and adrenergic blocking activity of a number of β -chloroethyl amines and with certain aspects of the mechanism of action of such compounds.

METHODS. *Mouse Protection Test.* The ability of adrenergic blocking agents to protect mice against the lethal effects of epinephrine has been utilized as a test for such activity (2). In the present study albino mice were injected subcutaneously with the compound to be tested and the LD₅₀₋₁₀₀ of epinephrine³ (20 mgm./kgm.) was subsequently injected intraperitoneally. Groups of 10 mice were used in each experiment and frequent control determinations of epinephrine toxicity were made. All injections were made in a volume corresponding to 0.1 cc./10 grams body weight, except in the case of epinephrine which was given in a volume of 0.2 cc./10 gram. The compounds tested were hydrochloride salts dissolved in normal saline (sometimes slightly acidified) or in propylene glycol diluted with normal saline. The number of mice per cage and the type of cage were kept constant.

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³ Commercially prepared 1:1,000 solution of the hydrochloride salt.

Blood pressure. Adult cats were anesthetized with 0.5 cc./kgm. of "Dial" solution (Ciba) administered intraperitoneally. The carotid artery was cannulated and the blood pressure recorded with a mercury or Hürthle manometer. All injections were made into the saphenous vein.

Nictitating membrane. The response of the nictitating membrane was recorded by attachment to an isotonic lever. Silver electrodes were placed on the cervical sympathetic trunk and stimulation effected by an inductorium.

Cross-circulation experiments. In 4 experiments cross-circulation was established in a pair of cats through glass cannulae which connected the cardiac end of the common carotid artery of each animal with the cardiac end of the external jugular vein of the other. Heparin was used as the anti-coagulant. Dibenamine was injected intravenously into 1 (donor) cat with the cross-circulation interrupted. After an interval, cross-circulation was established. Subsequent to this, with the cross-circulation again interrupted, the blood pressure response of the second (recipient) cat to epinephrine was recorded through a cannula in the femoral artery.

Isolated organs. A glass organ bath with a volume of 40 cc. was maintained at 38° C. A 4-5 cm. strip of freshly removed rabbit intestine or non-pregnant rabbit uterus was connected in the bath to a heart lever. A Krebs-Ringer bicarbonate medium was used containing 0.1 per cent glucose. A mixture of 95 per cent O₂ and 5 per cent CO₂ was bubbled continuously through the bath.

Papillary muscle. The technique of Cattell and Gold (4) was used. A Ringer-bicarbonate solution was gassed continuously with 95 per cent O₂ and 5 per cent CO₂. The muscle was stimulated at a rate of 1/sec. and responses were recorded photographically.

RESULTS. *Relationship of structure to adrenergic blocking activity.* A series of β -chloroethyl amines was studied for a protective effect against lethal doses (LD₅₀₋₁₀₀) of epinephrine in mice. Table 1 shows the results of 20 compounds so tested. After an initial dose of the β -chloroethyl amine (usually 20 mgm./kgm.) progressively smaller doses were tested until $\frac{1}{4}$ or more of a group of mice died from epinephrine. A few compounds reported previously (2, 3) are included for comparison. Those agents which failed to afford protection when epinephrine was given 30 minutes later were retested, allowing an interval of 2 hours between administration of the drug and of epinephrine.

Nickerson, Nomaguchi, and Goodman (3) reported that p-propyl or chlor substitution on the benzyl groups of dibenamine abolished activity, and that at least one benzyl group was essential for activity and could not be replaced by a phenyl, phenylethyl, or aliphatic group without complete inactivation. However, of the *bis* β -chloroethyl amines reported here, the benzyl, β -phenylethyl, and γ -phenylpropyl derivatives were active. Since benzhydrylethyl and β -biphenoxyethyl β -chloroethyl amines are also active, it appears that more than one C atom may be interposed between the N atom and the aromatic group in active compounds.

Of the agents tested for a protective effect in mice, α -naphthylmethyl ethyl β -chloroethyl amine appeared the most active. Any absolute comparison of the potency of these compounds in this regard would require the determination of the time taken for the development of maximal effect for each compound. This is evident in the case of γ -phenylpropyl *bis* β -chloroethyl amine which offered no protection when lethal doses of epinephrine were given 30 minutes later, but did protect against epinephrine administered 2 hours later. Thus any compari-

son of potency of adrenergic blocking agents reported herein is valid only for the particular time interval studied.

The finding that β -chloro β -phenylethyl amines may possess adrenergic blocking activity is of special interest. Dimethyl β -chloro β -phenylethyl amine was an active protective agent, the first demonstration that the aromatic group may be on the chloralkyl group in active compounds. This compound also showed striking cholinergic effects following intravenous administration in cats, causing: an acute flaccid paralysis, skeletal muscle fasciculations, a fall in blood pressure which was blocked by atropine, a depression of the responses of the intact cat gastrocnemius preparation to maximal motor nerve stimuli, and contraction of the nictitating membrane. This compound appears to undergo rapid cyclization at neutral pH (0.02 *M* solutions in 0.16 *M* NaHCO₃ attained maximal Cl⁻ evolution in 2 minutes) and its cholinergic effects may be ascribed to the resultant quaternary onium cation. These effects closely resemble those following the administration of methyl β -chloroethyl ethylenimonium ion to cats (5).

Evidence of adrenergic block in cats was manifest by reversal of blood pressure responses to injected epinephrine and by a reduction in the contractile responses of the nictitating membrane to cervical sympathetic stimulation. The latter was the more resistant to block. All of the compounds tested which had shown a protective effect in mice, caused some degree of adrenergic block in cats. There was little correlation of the potency on the 2 test objects, although larger doses were always required to induce adrenergic block in cats (table 1). In some cases reversal of the blood pressure responses to epinephrine was incomplete and the use of higher doses of the agent was prevented by its toxicity (dimethyl β -chloro β -phenylethyl amine). In general, all the adrenergic blocking agents tested blocked vasopressor responses to injected nor-epinephrine and epinephrine and had little effect on vasodepressor responses to epinephrine and isopropyl nor-epinephrine. In an occasional cat nor-epinephrine caused a slight fall in blood pressure after dibenamine.

Response to injected epinephrine was tested up to 30 minutes after a single dose of an agent. This would cause a low estimate of the potency of compounds which attain maximal effect more slowly than dibenamine.

Isolated tissues. Rabbit duodenum. As reported by Nickerson and Goodman (6), dibenamine had no effect on the reaction of this preparation to epinephrine. However in concentrations of 1:10,000 benzyl bis β -chloroethyl amine and α -naphthylmethyl bis β -chloroethyl amine prevented the inhibition of the spontaneous contractions caused by epinephrine. The significance of this *in vitro* effect is uncertain.

Rabbit uterus. Non-pregnant rabbit uterus showed contractile responses to epinephrine which were completely blocked by dibenamine (1:100,000 and less). This effect was resistant to repeated washing.

Papillary muscle. While Nickerson and Goodman (6) found that dibenamine had no effect on the positive inotropic action of epinephrine as judged by changes in pulse pressure, it was of interest to test this directly using the papillary muscle

technique. Dibenamine had no effect on the excitatory response to epinephrine, nor-epinephrine, or isopropyl nor-epinephrine (table 2). Benzyl bis β -chloro-

TABLE 1
Mortality of LD₅₀₋₁₀₀ of epinephrine in mice after certain 2-chloroethyl amines

R	R'	INT.*	DOSE (MG./KGM.)								ADRENERGIC BLOCK— CAT†
			20	10	5	2.5	1.0	0.5	0.25	0.01	
Mono-2-chloroethyl amines: $\begin{matrix} R \\ \\ N-CH_2CH_2Cl \\ \\ R' \end{matrix}$											
benzyl	benzyl ^{a, b}	30	0/30	0/10	5/10						10
o-methylbenzyl	o-methylbenzyl ^{a, b}	30	0/10	5/10							15 (inc.)
		120	10/10								
m-methylbenzyl	m-methylbenzyl ^{a, b}	30	0/10	0/10	4/10						
o-chlorobenzyl	o-chlorobenzyl ^{a, b}	30	10/10								
		120	9/10								
p-isopropylbenzyl	p-isopropylbenzyl ^{a, b}	30	9/10								
α -naphthylmethyl	ethyl ^c	30	0/20	0/10	0/10	0/20	0/10	0/10	3/20	8/10	5-6
methyl	methyl ^c	30	10/10								20 (inc.)
		120	10/10								
phenyl	ethyl ^d	30	7/10								
		120	9/10								
2-chloro, 2-phenylethyl amines: $\begin{matrix} R \\ \\ N-CH_2CH(Cl)-C_6H_5 \\ \\ R' \end{matrix}$											
methyl	methyl ^a	30	1/10	0/10	0/20	0/10	0/10	5/10			2 (inc.)
benzyl	benzyl ^{a, b}	30	10/10								
Bis 2-chloroethyl amines: $R-N=(CH_2CH_2Cl)_2$											
phenyl ^d		30	10/10								
		120	10/10								
benzyl ^b		30	3/10	2/10	0/10	0/10	0/10	0/10	4/10	8/10	8
β -phenylethyl ^d		30	0/10	3/10							10
γ -phenylpropyl ^d		30	10/10								
		120	1/10	0/10	1/10	0/10	1/10	5/10			
o-chlorobenzyl ^d		30	4/10	4/10	7/10						
p-chlorobenzyl ^{a, b}		30	7/10								
		120	9/10								
α -naphthylmethyl ^c		30	1/10	0/10	0/10	0/10	0/10	0/10	4/10		
p-nitrobenzyl ^{a, b, d}		30	10/10								
		120	9/10								
methyl ^{b, d}		30	7/10								

* Interval between administration of agent and injection of epinephrine.

† Approximate dose (mgm./kgm.) causing epinephrine reversal.

The author gratefully acknowledges samples of compounds from the following sources:

^a The Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Ind.

^b Chemical Corps, U. S. Army.

^c Parke, Davis and Company, Detroit, Michigan.

^d Merck & Company, Inc., Rahway, N. J.

ethyl amine was also without effect. These agents alone did not influence the contraction of the papillary muscle.

Duration of the adrenergic blockade. Cross-circulation. Experiments were performed in cats to test the possibility that circulating dibenamine might per-

sist for some time after intravenous administration. One of a pair of cats was given a dose of dibenamine intravenously which corresponded to 20 mgm./kgm.

TABLE 2

Response of the isolated papillary muscle to epinephrine after adrenergic blocking agents

MUSCLE	DRUG	CONCENTRATION	CHANGE IN RESPONSE (MM.)
#1	Epinephrine	1:1,000,000	10 → 24
	Benzyl bis β -chloroethyl amine + Epinephrine	1:500,000 1:1,000,000	10 → 28
	Benzyl bis β -chloroethyl amine + Epinephrine	1:250,000 1:1,000,000	12 → 32
#2	Epinephrine	1:1,000,000	7 → 22
	α -naphthylmethyl ethyl β -chloroethyl amine + Epinephrine	1:500,000 1:1,000,000	5 → 23
#3	Epinephrine	1:1,000,000	17 → 22
	Dibenamine + Epinephrine	1:500,000 1:1,000,000	8 → 22
	Dibenamine + Epinephrine	1:50,000 1:1,000,000	4 → 17

TABLE 3

Cross-circulation experiments in cats

EXPERIMENT	INTERVAL AFTER DIBENAMINE BEFORE MIXING	DURATION OF CROSS CIRCULATION	BLOOD PRESSURE RESPONSE OF RECIPIENT CAT. (MM.Hg) TO EPINEPHRINE (MICROGM/KGM.)		
			10	30	100
A	min.	min.			
	—	0	+66 (control)		
B	15	30	+26	+26	
	—	0	+66		
C	40	30	-20	-32 → +31	-26
	—	0	+106		
D	40	30	+20	+53	
	—	0	+100		
	270	30	+59		

of the weight of both cats. After an interval the circulations were allowed to mix, again interrupted, and the recipient cat tested for evidence of adrenergic block by the blood pressure response to injected epinephrine. The results are shown in table 3. Definite evidence of adrenergic block in the recipient cat was

obtained 15 and 40 minutes after the administration of dibenamine to the donor, and in one cat an effect was still present after 4.5 hours. Mixing of a dye which is confined to the vascular space is complete in this preparation after 5 minutes of cross circulation (7). Cross circulation alone had no effect on the blood pressure at about the same level. These findings indicate that circulating dibenamine may persist for several hours after a single dose.

Protection experiments in mice. Additional evidence for the persistence of dibenamine is provided by experiments which utilized the ability of thiosulfate ion, when administered prior to dibenamine, to prevent the development of adrenergic block. Thiosulfate ion is distributed to the extracellular space and is rapidly cleared by the kidney (8). Cats given sodium thiosulfate 0.5

TABLE 4

*Mortality of LD₅₀₋₁₀₀ of epinephrine in mice receiving sodium thiosulfate followed by adrenergic blocking agents**

INTERVAL BETWEEN AGENT AND EPINEPHRINE (MIN.)	DIBENAMINE		α -NAPHTHYLMETHYL ETHYL β -CHLOROETHYL AMINE		DIMETHYL β -CHLORO β -PHENYLETHYL AMINE	
	Dose†	Mortality	Dose	Mortality	Dose	Mortality
30	20	16/20	20 1.0 0.25	0/10 0/10 7/10	1.0	9/10
60	20	8/10				
120	20	0/10	20 1.0 0.25	0/10 0/10 9/10	1.0	10/10
240	20	2/10				

* Sodium thiosulfate 1.0 gm./kgm. administered subcutaneously 30 min. prior to adrenergic blocking agent. Dose of epinephrine 20 mgm./kgm.

† mgm./kgm.

gram/kgm. intravenously, followed by dibenamine 20 mgm./kgm. intravenously, showed pressor responses to injected epinephrine when tested 30 minutes later, but after an interval of 2 hours showed typical epinephrine reversal. Similarly, mice which received sodium thiosulfate followed by dibenamine showed no protection against the lethal effects of epinephrine when tested 30 or 60 minutes later (table 4). However, when epinephrine was given 2 and 4 hours after dibenamine in similar experiments protection was evident. This delayed epinephrine protection is manifest after an interval allowing for excretion of thiosulfate and indicates the slow transformation of dibenamine *in vivo*. Similar thiosulfate experiments with α -naphthylmethyl ethyl β -chloroethyl amine and dimethyl β -chloro β -phenylethyl amine failed to show this delayed protection (table 4) and indicate a more rapid transformation *in vivo* of these compounds than of dibenamine.

The ability of thiosulfate to prevent the adrenergic blocking effect of α -naphthyl-

methyl ethyl β -chloroethyl amine is limited. Thus, thiosulfate prevented the protective effect of 0.25 mgm./kgm. of this agent but did not alter the effect of larger doses (table 4). Similarly in cats, even after large doses of thiosulfate (1 gram/kgm.) adrenergic block sometimes followed the injection of dibenamine, 20 mgm./kgm., administered 5 minutes later.

Thiosulfate had no effect on the protective action of dibenamine in mice when given subsequent to the dibenamine. Likewise cats which had received dibenamine 20 mgm./kgm. showed no change in the adrenergic block when large amounts of sodium thiosulfate were given.

The duration of the protection afforded mice against the lethal effects of epinephrine was compared for 3 adrenergic blocking agents (table 5). The duration of effect of both dibenamine and α -naphthylmethyl ethyl β -chloroethyl amine was similar while dimethyl β -chloro β -phenylethyl amine showed a less prolonged action.

TABLE 5

Mortality of LD₅₀₋₁₀₀ of epinephrine administered to mice at varying intervals after adrenergic blocking agents

INTERVAL BETWEEN AGENT AND EPINEPHRINE	DIBENAMINE		α -NAPHTHYLMETHYL ETHYL β -CHLOROETHYL AMINE		DIMETHYL β -CHLORO β -PHENYLETHYL AMINE	
	Dose*	Mortality	Dose	Mortality	Dose	Mortality
30 min.	20	0/10	0.5	0/10	2.0	0/10
5 hr. 30 min.	20	0/10	0.5	0/10	2.0	4/10
13 hr.	20	0/10	5	1/10	5	9/10
24 hr.	20	0/10	0.5	8/10	2.0	9/10
40 hr.	20	8/10	0.5	8/10		

* mgm./kgm.

DISCUSSION. A considerable number of β -chloroethyl amines have been shown to possess adrenergic blocking activity. The specificity of structure of active agents is not striking but all are tertiary substituted β -halogenated amines in which at least one of the substituted groups is an aromatic ring separated by at least one C atom from the N atom. The following aromatic groups have been present in active compounds: phenyl, α -naphthyl, benzhydryl, and biphenoxy (1, 2). Substitution on such aromatic groups may reduce activity by altering the ability of the compound to undergo cyclization. Comparisons of the relative potency of such compounds is rendered difficult by the fact that maximal effects are reached at different rates. However, of the agents studied thus far, the α -naphthylmethyl β -halogenated ethyl amines appear the most potent.

The aromatic group may be located on the chloralkyl group in an active compound as evidenced by dimethyl β -chloro β -phenyl ethyl amine. While this compound is too cholinergic to possess therapeutic potentialities, the possibility exists that analogues in which the methyl groups were replaced by higher alkyl radicals might have less of these undesirable effects.

Evidence is presented which indicates that dibenamine is transformed slowly *in vivo*. However, α -naphthylmethyl ethyl β -chloroethyl amine appears to undergo more rapid transformation *in vivo* and yet has an action as prolonged as that of dibenamine. The marked insolubility of dibenamine at physiological pH may be an important factor in determining its reactivity. Because of these solubility characteristics it is probable that dibenamine has considerable lipoid affinity *in vivo*. The slow rate of transformation of dibenamine does not appear responsible for its prolonged action. Rather, there is good evidence that the union between dibenamine and the effector cell is of long duration. Thus, large doses of thiosulfate do not alter the adrenergic block once dibenamine has been given. *In vitro*, the effect of dibenamine in blocking excitatory responses of the non-pregnant rabbit uterus to epinephrine is resistant to repeated washing. The nature of this prolonged effect upon the effector cell may be due to a firm fixation at a receptor surface (perhaps analogous to atropine), or, as suggested by Nickerson and Goodman (6), to the irreversible inactivation of some hypothetical enzyme system essential for excitatory responses of effector cells to epinephrine or excitatory sympathins. Both require the additional explanation of why excitatory responses of the heart to injected epinephrine or to splanchnic stimulation persist after dibenamine (6). The implication is that adrenergic receptors of the heart differ from receptors elsewhere in muscle effectors which respond to epinephrine by excitation. This is supported by the finding that isopropyl nor-epinephrine, which is exclusively vasodepressor, causes excitation of the heart. Changes in heart rate may thus fail to be purely a measure of the release of excitatory sympathin.

SUMMARY

1. A series of β -chloroethyl amines has been studied for evidence of adrenergic blocking activity by their ability to protect mice against lethal doses of epinephrine. Active agents were tested for the production of adrenergic block in cats.

2. From data reported herein and elsewhere, it appears that activity resides in tertiary β -halogenated amines substituted with certain aromatic groups separated by at least one C atom from the N atom.

3. Evidence is presented that dibenamine is transformed slowly *in vivo*. This does not appear to be responsible for the prolonged action of this and related compounds.

4. The absence of an effect of such adrenergic blocking agents upon the excitatory response of the heart to epinephrine is confirmed using the papillary muscle technique.

REFERENCES

1. NICKERSON, M., AND GOODMAN, L.: Fed. Proc., 5: 194, 1946.
2. LOEW, E. R., AND MICETICH, A.: Fed. Proc., 6: 351, 1947.
3. NICKERSON, M., NOMAGUCHI, G., AND GOODMAN, L.: Fed. Proc., 5: 195, 1946.
4. CATTELL, McK., AND GOLD, H.: THIS JOURNAL, 62: 116, 1938.
5. HUNT, C. C., AND PHILIPS, F. S.: THIS JOURNAL, to be published.
6. NICKERSON, M., AND GOODMAN, L.: THIS JOURNAL, 89: 167, 1946.
7. CLARK, D.: Personal Communication.
8. GILMAN, A., PHILIPS, F. S., AND KOELLE, E. S.: Am. J. Physiol., 146: 348, 1946.

THE ANTIDIURETIC EFFECT OF 3-HYDROXY-CINCHONINIC ACID DERIVATIVES¹

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The present communication presents data on the antidiuretic effect of certain derivatives of cinchoninic acid. A number of these same cinchoninic acid derivatives have been found to cause an increased excretion of uric acid in man (1, 2.)

METHODS. The antidiuretic effect of the compounds was tested on a water diuresis in the dog. Female dogs were used. These had been trained and were accustomed to the passage of a stomach tube, catheterization, and venipuncture. Several of the animals were subjected to perineotomy in order to facilitate catheterization. Diuresis was produced by giving about 40 cc. of water per kilogram by mouth. Usually, urine was obtained by catheter every fifteen minutes. After diuresis had been established an intravenous injection of one of the compounds was given and the effect on diuresis noted. Precautions were taken to avoid the inhibition of water diuresis produced by emotional stress or afferent nerve stimulation (3, 4, 5).

All compounds were first given in a dose of 20 milligrams per kilogram; a solution of the sodium salt was used. If effective, smaller doses were then used. All compounds have been tested on at least two dogs.

Creatinine clearance was determined in the usual manner. Creatinine was given subcutaneously about one-half hour and water one-half and one hour before starting clearance measurements. Creatinine was determined by the Folin method (6). Clearance periods varied from 10 to 22 minutes in length.

PREPARATION OF COMPOUNDS. All of the cinchoninic acid derivatives other than the 2-phenyl derivative (cinchophen) were prepared in this laboratory. Quininic acid (6-methoxycinchoninic acid) was obtained by saponification of ethyl quinate kindly supplied by Dr. Joseph B. Koepfli. New methods for the synthesis of 3-hydroxy-2-methyleinchoninic acid and of 3-hydroxy-2-phenyleinchoninic acid were devised to facilitate the preparation of these substances in quantity. These methods are described below. The remainder of the cinchoninic acids were prepared by established methods as follows: 2-hydroxy-3-phenyleinchoninic acid by the method of Gysae (7), 2-hydroxycinchoninic acid by the method of Borsche and Jacobs (8), 2-hydroxy-3-methyleinchoninic acid by the method of Meyer (9), 2-methyleinchoninic acid by the method of Pfizinger (10) and both quinaldine-3,4-dicarboxylic acid and quinoline-2,3,4-tricarboxylic acid by the method of Mitchovitch (11).

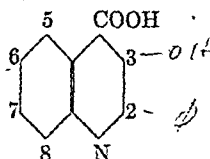
3-Hydroxyquinaldine-4-carboxylic Acid. This substance has previously been prepared by diazotization of 3-aminoquinaldine-4-carboxylic acid (Berlingozzi and Marzella, 12) and by dimethylation of 3-methoxyquinaldine-4-carboxylic acid (Diethey and Thelen, 13). Isatin (73.5 gm., 0.5 mole) was added with shaking to a cold solution of 350 gm. of 97.7 per cent sodium hydroxide in 1000 cc. of water contained in a 3 liter flask. When the isatin had dissolved, 70 gm. (20 per cent in excess of the theoretical amount) of technical acetol acetate were added and the mixture was heated under reflux in an actively boiling water bath for 6 hours, then cooled to room temperature by immersion of the reaction flask in running water. Concentrated hydrochloric acid (approximately 600 cc.) was then added until precipitation of the product began, then 100 cc. of glacial acetic acid were added. After standing overnight at room temperature, the product was collected on a 150 mm. Buchner funnel, washed with four 75 cc. portions of water and sucked as dry as possible. The crude product was suspended in 1200 cc. of water, brought into solution by the addition of 40 cc.

¹ This investigation has been aided by a grant from the U. S. Public Health Service.

of 28 per cent ammonia water and the resulting solution filtered. To the filtrate, 110 cc. of 6 N acetic acid were added slowly with continuous stirring. After several hours at room temperature, the product was collected on a Buchner funnel, washed with six 50 cc. portions of water, sucked as dry as possible, air dried for a few days and then dried to constant weight at 60°. Yield, 91.4 gm. (90 per cent of the theoretical yield) of buff colored microcrystalline powder, m. pt. 204-206° with gas evolution.

3-Hydroxy-2-phenylcinchoninic Acid. This substance has previously been obtained by the Pfitzinger condensation of isatin with phenacyl bromide. In our experience, the substitution of phenacylacetate for the halide and the use of less concentrated alkali for the conduct of the condensation results in markedly enhanced yields of cleaner material than s obtained by the previously published methods of synthesis (14, 15).

TABLE 1
Antidiuretic effect of Cinchoninic acids



NO.	SUBSTITUENTS	EFFECT
1	2-methyl-3-hydroxy	+
2	2-hydroxy-3-methyl	-
3	2-methyl	-
4	2-methyl-3-carboxy	-
5	2-hydroxy	-
6	6-methoxy	-
7	2-3-dicarboxy	-
8	2-phenyl-3-hydroxy	++
9	2-hydroxy-3-phenyl	++
10	2-phenyl	-

The minimal quantity of a solution of 83 gm. of 97.7 per cent sodium hydroxide in 275 cc. of water necessary to effect solution was added to a suspension of 73.5 gm. (0.5 mole) of isatin in 600 cc. of water contained in a 3 liter flask. To the solution obtained, there was added first, a solution of 90.8 gm. (0.51 mole) of phenacyl acetate in 500 cc. of warm ethanol and then the remainder of the solution of sodium hydroxide. The mixture was refluxed over a free flame for three hours and allowed to remain at room temperature overnight. It was then diluted with 1300 cc. of water and filtered to remove tarry material. To the filtrate with constant stirring there was added in order 165 cc. of concentrated hydrochloric acid and 55 cc. of glacial acetic acid. After remaining at room temperature overnight, the precipitated product was collected upon a 150 mm. Buchner funnel, washed with four 50 cc. portions of cold water and transferred to a 4 liter beaker containing 1500 cc. of water. The solid was brought into solution by the addition of 40 cc. of 28 per cent ammonia water and filtered from a slight amount of insoluble matter. To the filtrate 100 cc. of 6 N acetic acid were added and, after several hours at room temperature, the product was separated by filtration, washed with four 75 cc. portions of water, dried at room temperature for a few days and then at 60° until constant weight was attained. Yield, 108.7 gm. (82 per cent of the theoretical) of deep yellow microcrystalline product, m. pt. 206-207° with decomposition.

RESULTS. Ten cinchoninic acid derivatives were tested. Results are given in table 1. Only two of the compounds exhibit an antidiuretic effect in an intra-

venous dosage of 20 milligrams per kilogram. Of these two, the phenyl derivative appears to be more potent than the methyl derivative. Data of experiments with three doses of the 3-hydroxy-2-phenylcinchoninic acid are given in figure 1. In figure 2, data on the effect of the same compound are given; here rate of urine flow was measured every two minutes.

The relation of rate of urine flow and creatinine clearance for the two active compounds is shown in table 2. The creatinine clearance appears to be unchanged during the periods of lowest rate of urine flow.

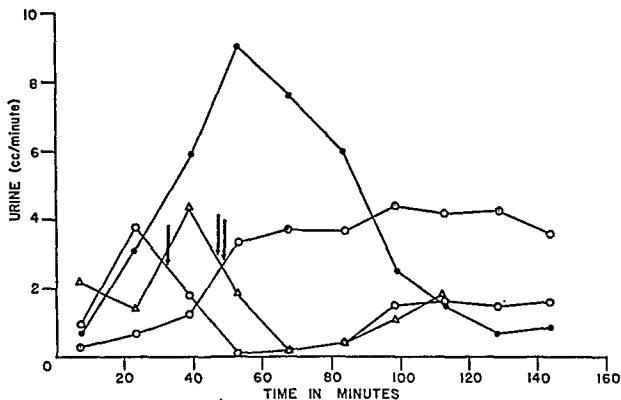


FIG. 1. EFFECT OF 3-HYDROXY-2-PHENYLCINCHONINIC ACID ON WATER DIURESIS IN DOG—BROWNHEAD HOUND, 17.5 KGM.

At zero minutes, 40 cc. of water per kgm. given by mouth. Rate of urine flow plotted at middle of collection period. At arrows drug given intravenously on different days. —●—●— control, no drug; —○—○— 20 mgm./kgm.; —△—△— 8 mgm./kgm.; —○—○— 3 mgm./kgm.

Experiments were done to test the efficacy of oral administration of one of the drugs. Figure 3 gives the data of one of these experiments.

The 3-hydroxy-2-phenylcinchoninic acid has been tested on two "neurohypophysectomized" dogs. In two experiments on one dog a marked antidiuretic effect on a water diuresis was obtained; in two experiments on the other dog a slight antidiuretic effect was observed. We are not reporting the results in detail because complete lack of posterior hypophyseal tissue has not yet been confirmed on these dogs and because further experiments on "neurohypophysectomized" dogs are in progress. This derivative has also been found to produce a definite reduction in the polyuria and polydipsia when given by mouth to several cases of diabetes insipidus.²

² We wish to thank Dr. Richard de Bodo of the Department of Pharmacology, New York University, for performing these preliminary experiments on his "neurohypophysectomized" dogs. Three cases of diabetes insipidus were studied here. We are indebted to Dr. Stanley E. Bradley of the Presbyterian Hospital for giving the drug to other cases. These studies are being continued and will be reported in detail later.

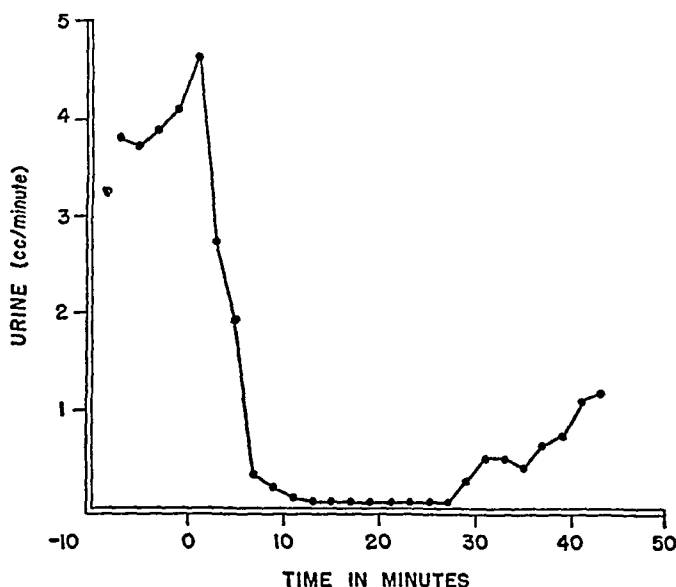


FIG. 2. EFFECT OF 3-HYDROXY-2-PHENYLCINCHONINIC ACID ON WATER DIURESIS IN DOG—SPOT, 12 KGM.

Observations started one hour after administration of 40 cc. water per kilogram by mouth. Urine flow recorded every two minutes. At zero minutes, intravenous injection of 3 mgm./kgm. of drug.

TABLE 2
Urine flow and creatinine clearance

DRUG* NO.	DOSE		1	2	3	4	5	6	7
	mgm./ kgm.								
1	20	Urine Flow† Clearance†	1.17 121	2.45 107	1.78† 97	0.36 102	0.44 105	1.30 104	2.50 102
1	10	Urine Flow Clearance	7.90 103	7.21 92	2.32† 80	0.62 95	2.78 101		
8	10	Urine Flow Clearance	8.20 122	8.00 97	0.78† 60	0.40 89	0.38 91		
8	10	Urine Flow Clearance	0.85 60	2.57 75	3.57 76	1.06† 47	0.20 69	0.44 68	

* See Table 1.

† Both rate of urine flow and creatinine clearance are expressed as cc. per minute.

‡ Drug injected intravenously just before start of this period.

DISCUSSION. An inhibition of water diuresis may be caused by either a decrease of glomerular filtrate (due generally to circulatory changes) or an increased reabsorption of water by the tubule. The first type of inhibition is usually rapid in onset and of short duration, while the second type is slower in onset and of longer duration than the first (5). This suggests that the inhibition

caused by the cinchoninic acid derivatives belongs to the second type. That this is so is shown by an examination of the creatinine clearances. If it is assumed that the creatinine clearance measures glomerular filtrate in the dog, it is clear that the rate of glomerular filtration is not appreciably changed during the inhibition of diuresis caused by the drug. However, a decrease in the creatinine clearance may occur in the first period after intravenous injection of drug; this occurs before the most marked decrease in rate of urine flow, and may be due to a secondary transient vascular effect of the drug.

A number of drugs besides posterior pituitary extract have been found to inhibit water diuresis. Examples are: β -imidazolethylamine and β -oxyphenyl-

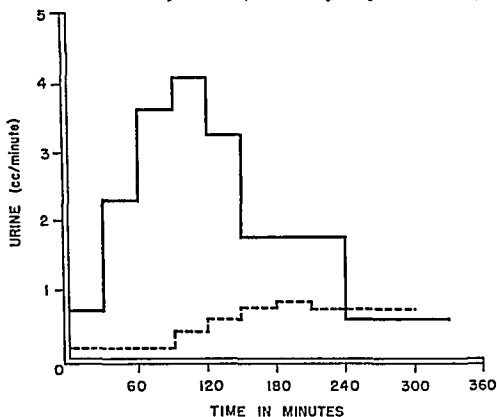


FIG. 3. EFFECT OF 3-HYDROXY-2-PHENYLCINCHONINIC ACID ON WATER DIURESIS IN DOG WHEN GIVEN ORALLY. DOG—BROWNIE, 18 KGm.

At zero minutes, 700 cc. of water by mouth. ——— control, no drug. — — — 30 mgm./kgm. of drug given in gelatin capsules fifteen minutes before administration of water.

ethylamine (16), atropine (17, 18, 19), yohimbine (20, 21), choline (22), acetylcholine (22, 23), morphine (24), phenobarbital (25), and nicotine (26). However, of these all which have been investigated as to their mechanism of action (yohimbine, acetylcholine, nicotine, morphine and phenobarbital) appear to produce their antidiuretic effect by an action on the central nervous system causing liberation of the antidiuretic hormone of the posterior pituitary gland. The 3-hydroxy-2-phenylcinchoninic acid appears to act quite differently in that it appears to be effective in neurohypophysectomized dogs and in cases of diabetes insipidus.

SUMMARY

A number of cinchoninic acid derivatives have been examined for their antidiuretic effect in the dog. The 3-hydroxy-2-methyl and 3-hydroxy-2-phenylcinchoninic acids have an inhibitory effect on a water diuresis.

REFERENCES

- 1) NICOLAIER, A., AND DOHRN, M.: *Deutsches Arch. f. klin. Med.*, **93**: 331, 1908.
- 2) BERLINGOZZI, S., AND DONATELLI, G.: *Boll. Chim. Farm.*, **75**: 387, 1936.
- 3) THEOBALD, G. W.: *J. Physiol.*, **81**: 243, 1934.
- 4) THEOBALD, G. W., AND VERNEY, E. B.: *J. Physiol.*, **83**: 341, 1935.
- 5) O'CONNOR, W. J., AND VERNEY, E. B.: *Quart. J. Exper. Physiol.*, **33**: 77, 1946.
- 6) War Department Technical Manual (TM-227): *Methods for Laboratory Technicians*, p. 196.
- 7) GYSAE, G.: *Ber.*, **26**: 2478, 1893.
- 8) BORSCHKE, W., AND JACOBS, W.: *Ber.*, **47**: 354, 1914.
- 9) MEYER, H.: *Monatsch.*, **26**: 1311, 1905.
- 10) PFITZINGER, W.: *J. prakt. Chem.*, (2) **33**: 100, 1886.
- 11) MITCHOVITCH, V. M.: *Bull. soc. Chim.*, (5) **6**: 1156, 1939.
- 12) BERLINGOZZI, S., AND MARZELLO, C.: *Atti accad. Lincei*, (v) **32**: ii, 403, 1923.
- 13) DIETHEY, W., AND THELEN, C.: *Ber.*, **58**: 1588, 1925.
- 14) BERLINGOZZI, S., AND CAPUANO, G. B.: *Atti accad. Lincei* (v) **33**: ii, 91, 1924.
- 15) JOHN, H., AND FRÄNKEL, R.: *J. prakt. Chem.*, **133**: 259, 1932.
- 16) MOTZFELDT, K.: *J. Exper. Med.*, **25**: 153, 1917.
- 17) GINSBERG, W.: *Arch. f. exper. Path. u. Pharmacol.*, **69**: 381, 1912.
- 18) COW, D.: *Arch. f. exper. Path. u. Pharmacol.*, **69**: 393, 1912.
- 19) SSARGIN, K. D., AND NUSSINBOIM, B. E.: *Arch. internat. pharmacodyn. et de therap.*, **57**: 195, 1937.
- 20) ZUNZ, E., AND VESSELOVSKY, O.: *Compt. rend. Soc. de biol.*, **131**: 135, 1939.
- 21) FUGO, N. W.: *Endocrinology*, **34**: 143, 1944.
- 22) MOLITOR, H., AND PICK, E. P.: *Arch. f. exper. Path. u. Pharmacol.*, **101**: 198, 1924.
- 23) PICKFORD, M.: *J. Physiol.*, **95**: 226, 1939.
- 24) DE BODO, R. C.: *THIS JOURNAL*, **82**: 74, 1944.
- 25) DE BODO, R. C.: *THIS JOURNAL*, **85**: 222, 1945.
- 26) BURN, J. H., TRUELOVE, L. H., AND BURN, I.: *Brit. Med. J.*, **1**: 403, 1945.

γ -DICHROINE, THE ANTIMALARIAL ALKALOID OF CH'ANG SHAN¹

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In China, for many centuries, a medicinal herb called Ch'ang Shan has been known for its antimalarial action (2-3). Its emetic and cathartic effects have also been described. The root is employed in medicine, although the leaves are also believed active. The above-mentioned Chinese references give directions how to make a tea, a wine, or pills of the dried root of Ch'ang Shan, for the control of various forms of malaria. The plant, an evergreen shrub, is indigenous to Southwestern China. After the introduction of quinine in China, Ch'ang Shan gradually became a remedy of only local interest—namely in Yunnan and Szechuan Provinces.

There is much confusion about the botanical identity of Ch'ang Shan. Some authors named it *Oriza japonica* (4-5), while others called it *Dichroa febrifuga* (6). Still other terminology was discussed by Yang (7). Although there has been no final verification, most investigators accept *Dichroa febrifuga* as the correct name. The pharmacognosy of Ch'ang Shan and its cultivation have recently been presented by Yu (8).

Several Japanese workers became interested in Ch'ang Shan, and studied its history, histology, and geographical distribution (9-10). Terada and his co-workers failed to show the efficacy of the infusion of Ch'ang Shan in the *Plasmodium* infection of canaries (11). They, too, ran into controversy over its botanical identity (9, 12-13). Since Terasaka's chemical investigation (13) was carried out with the Japanese variety of *Oriza japonica*, his results do not apply to Ch'ang Shan.

During the Japanese occupation of Eastern China in World War II, the Chinese government was forced to move to Chungking, a city in Southwestern China. With it, was the migration of large masses of people from coastal and central provinces. Unfortunately, Southwestern China is a pandemic area of different forms of malaria. A large portion of the new population, including the governmental and military personnel, fell victims of malaria, and sustained a high mortality rate. Meanwhile, the Dutch East Indies were lost to the Japanese, resulting in a cut-off of 90 per cent of the world's supply of quinine to the allies. Under this desperate condition, the Chinese immediately directed their attention to crude drugs of their own, including Ch'ang Shan.

Scientific studies were initiated in Chinese governmental laboratories. Although the preliminary note of Liu and his associates (14) was not enlightening, their results with the crude powder in humans seemed to indicate that the herb

¹ Read in part at the Fall Meeting of the American Physiological Society at Minneapolis, Minnesota, on September 17, 1948 (1).

was efficacious in the treatment of tertian malaria. The work was continued at the National Institute of Health by Jang and his colleagues (6, 15-17), and the therapeutic effect of the extract of Ch'ang Shan in 13 clinical cases was confirmed. Progress was made when Chou and his co-workers (18-19), at the Institute of Materia Medica, Shanghai, announced the isolation of 3 isomeric alkaloids in crystalline form, of which γ -dichroine was the most active in *gallinaceum* infection of chickens.

In the United States, the Board for the Coördination of Malarial Studies became interested in Ch'ang Shan, and assigned to it the survey number SN-10,767 (20). In coöperation with the Board, our laboratories purchased in the fall of 1942 360 pounds of Ch'ang Shan from Chinese drug shops in different localities—Honolulu, San Francisco, Vancouver, B. C., Chicago, New York City, and Havana. In addition, we imported 500 pounds from Chungking, China, by an American military plane. Koepfli, Mead, and Brockman (21), using the extracts we prepared, succeeded in isolating 2 isomeric alkaloids, one of which, febrifugine, is about 100 times as active as quinine (Q value of 100) against *P. lophurae* in ducks. Kuehl, Spencer, and Folkers (22), working independently, also obtained 2 isomeric alkaloids having Q values of 8 and 16, respectively, against *P. gallinaceum* in chicks. It is interesting that the proposed empirical formula of the two groups of American investigators is the same, namely, $C_{16}H_{15}O_2N_3$. It also agrees with the formula of the Chinese workers, published in their preliminary note (18), but differs from the revised formula of their second paper (19) by 2 atoms of hydrogen.

Ch'ang Shan has been studied in England by Tonkin and Work (23) and Hooper (24). The latter separated a glucoside, but failed to find any alkaloids.

Dr. T. Q. Chou, Director of the Institute of Materia Medica, Shanghai, made available to us a generous supply of γ -dichroine. Our study was limited to the estimation of its antimalarial activity, determination of its activity, and assessment of its effects on blood pressure, respiration, gastrointestinal tract, blood sugar, and body temperature. The material was crystalline-white, was in needle-form under the microscope, and melted at 160°C . (corrected). The alkaloid required dilute acid for solution—a volume of 3.42 cc. of N/100 HCl for every 10 mgm. of γ -dichroine. For pharmacologic experiments, a fresh stock solution of 0.1 per cent was prepared.

1. *Antimalarial Activity.* (a) *Ducks.* The method of study was similar to I-2 already described (25), with certain modifications. Briefly, ducklings, 4-weeks-old, were infected with *P. lophurae* by transfusion of blood from another duckling previously infected with the same organism. The infecting dose was 2 billion parasitized red cells per kgm. of body weight. Each animal was treated with γ -dichroine by intravenous injection within the first 4 hours, and again within 8 hours, after inoculation. The medication was continued for 5 days, 3 times a day. On the sixth day, parasitized erythrocytes were counted on thin films. Control animals with quinine, and without any medication, were run at the same time. A total of 134 ducklings were used.

The results are summarized in table 1. It should be observed that ducklings

without treatment developed an average parasitemia of 80 per cent. The dose which caused 40 per cent parasitemia was therefore considered the median suppressive dose (SD_{50}), and was calculated from the straight line fitted to the data by the method of least squares when average parasitemia in per cent was plotted against the log dose. The standard error in each case was omitted. As shown in table 1, the SD_{50} for γ -dichroine is 0.064, and that for quinine 9.490, mgm. per kgm. The Q value of γ -dichroine, namely, the ratio between 9.49 and 0.064, is therefore 148.

TABLE 1
Antimalarial activity of γ -dichroine in birds

ANIMAL	Plasmodium	DRUG	DOSE	NUMBER OF ANIMALS USED	AVERAGE PARASITEMIA	SD_{50}
			mgm./kgm.		%	mgm./kgm.
Duckling	<i>lophurae</i>	γ -Dichroine	0.10	5	6.16	0.064
			0.09	5	23.40	
			0.08	5	30.00	
			0.07	5	46.20	
			0.06	5	18.40	
			0.05	5	66.40	
		None	—	20	80.00	9.490
		Quinine	12.00	21	11.61	
			10.00	33	38.39	
			7.50	30	64.57	
Canary	<i>relictum</i>	γ -Dichroine	0.16	9	14.00	0.146
			0.11	18	18.11	
			0.08	5	28.20	
		None	—	19	28.57	20.000
		Quinine	20.00	13	14.69	
			16.00	5	29.20	

(b) *Canaries*. A similar test was carried out in canaries infected with *P. relictum*² according to our procedure I-1 (25), with the exception that medication was given by vein and continued for 5 days, and that parasitized erythrocytes were counted on the sixth day. A total of 69 birds were employed. It will be noted that the SD_{50} of γ -dichroine is 0.146, and that of quinine 20, mgm. per kgm. The Q value of γ -dichroine in canaries against *P. relictum* is therefore 137.

(c) *Monkeys*. Dr. Leon H. Schmidt, Director of the Institute of Medical Research, Christ Hospital, Cincinnati, kindly administered γ -dichroine by

² We are indebted to Dr. Clay G. Huff, Chief Parasitologist, Naval Medical Research Institute, National Naval Medical Center, Bethesda 14, Maryland, for his generous supply of this organism.

stomach tube to rhesus monkeys infected with *P. cynomolgi*. Since Dr. Schmidt will publish his results in detail, it suffices to mention that doses of γ -dichroine ranging from 0.4 to 0.8 mgm. per kgm. definitely reduced the parasitemia, and one of 1.6 mgm. per kgm. cleared the blood stream of parasites to such an extent that a thick film showed no count (26).

2. *Toxicity.* The acute toxicity of γ -dichroine was determined in starved albino mice by both intravenous and oral administration. These studies were made on the same day, with the same solution, and on the same group of mice. Death occurred in 48 to 72 hours. As shown in table 2, the median lethal dose (LD_{50}) by mouth is 2.74 ± 0.41 , and by vein, 10.0 ± 0.50 , mgm. per kgm. It is of particular interest that this alkaloid is approximately $3\frac{1}{2}$ times as toxic when given orally than by vein.

Repeated intravenous injections at different dose levels were given to mice in an attempt to obtain some knowledge of any pathology which might occur. Necropsy was performed immediately following the death of the animal. Table 3

TABLE 2
The acute toxicity of γ -dichroine in mice

ROUTE OF ADMINISTRATION	DOSE	NUMBER DIED NUMBER USED	$LD_{50} \pm S. E.$
	mgm./kgm.		mgm./kgm.
Intravenous	8.0	1/10	10.0 ± 0.5
	10.0	5/10	
	12.5	9/10	
	16.0	10/10	
Oral	0.625	0/10	2.74 ± 0.41
	1.25	2/10	
	2.5	2/10	
	5.0	9/10	
	8.0	10/10	

summarizes the results of this work. Of the 27 animals which came to necropsy, one animal showed parenchymatous degeneration of the kidney with some necrosis. Twelve animals (44.4 per cent) showed hydrops of the liver characterized by large, vacuolated hepatic cells, as illustrated in figure 1. The same lesion occurred after γ -dichroine was injected subcutaneously or intraperitoneally (table 3).

3. *Emetic Action.* γ -Dichroine produced vomiting in pigeons. When it was administered by vein to starved pigeons, the initial episode of vomiting occurred in 15 to 30 minutes. This was followed by repeated attacks of vomiting at 5- to 10-minute intervals. The incidence of vomiting with different doses over the number of birds used is as follows: $\frac{2}{3}$ on a dose of 0.1, $\frac{2}{3}$ on one of 0.2, and $\frac{4}{5}$ on one of 0.3, mgm. per kgm. The median emetic dose (EmD_{50}) is 0.132 ± 0.04 mgm. per kgm. It would appear that γ -dichroine is responsible for vomiting in man when Ch'ang Shan or its extract is used in the treatment of malaria (2, 3, 6, 14, 15).

4. *Effect on Respiration and Circulation.* Because of the small amount of material available, studies on respiration and circulation were limited. Dogs under 'Sodium Amytal' (Sodium Iso-amyl Ethyl Barbiturate, Lilly) anesthesia

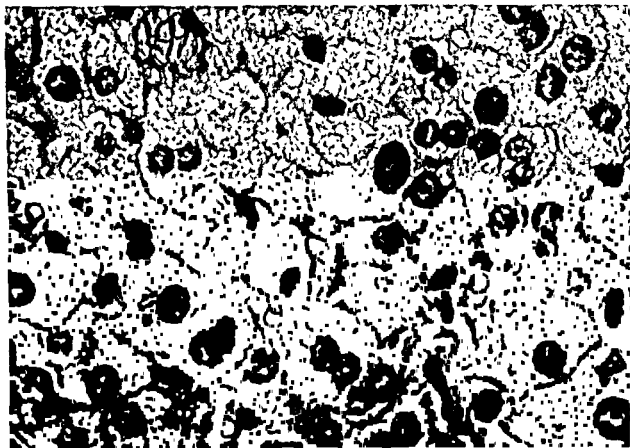


FIG. 1. HYDROPIC DEGENERATION OF THE LIVER

Mouse 1, weighing 23.5 gm., received a daily dose of 4 mgm. per kgm. of γ -dichroine by intraperitoneal injection. It died following the eighth dose.

TABLE 3
Results of repeated injections of γ -dichroine in mice

DOSE	NUMBER OF ANIMALS	AVERAGE NUMBER OF INJECTIONS PER MOUSE	ROUTE OF ADMINISTRATION	NUMBER TO NECROPSY	PATHOLOGY
mgm / kgm.					
2	5	14	Intravenous	5	1 Hydrops of liver; 1 parenchymatous degeneration of kidneys
4	5	8	Intravenous	4	4 Pulmonary edema and atrophy of thymus
8	5	4	Intravenous	4	2 Hydrops of liver
10	10	2	Intravenous	5	All showed minimal or slight hydrops of liver
4	5	8	Subcutaneous	4	2 Hydrops of liver
4	5	7	Intraperitoneal	5	2 Hydrops of liver

showed a fall in blood pressure of 20 mm. Hg following the intravenous injection of 4 mgm. per kgm. There was a transient increase in respiratory rate. Smaller doses, such as one of 2.5 mgm. per kgm., produced very slight changes in both blood pressure and respiration, as exemplified in figure 2.

5. *Action on Intestine.* As shown in figure 2, a dose of 2.5 mgm. per kgm. of γ -dichroine caused an increase in duodenal peristalsis in anesthetized dogs. The duodenal movements were recorded by a balloon through a Harvard membrane manometer. The increase in activity began 5 to 10 minutes following injection and lasted for 2 to 3 hours. This picture was duplicated in 3 other dogs. Following decerebration and pithing, and also double vagotomy, the alkaloid produced the same stimulation of intestinal activity. With atropinization, the stimulating action of γ -dichroine was inhibited for a short time.

In 4 non-anesthetized rabbits, doses of 5 and 10 mgm. per kgm. of the alkaloid induced marked diarrhea in 30 minutes following intravenous injection, which persisted for 5 to 6 hours. Ten non-anesthetized rats also developed pronounced diarrhea—each following a dose of 2.5 mgm. per kgm., 5 by intravenous injection

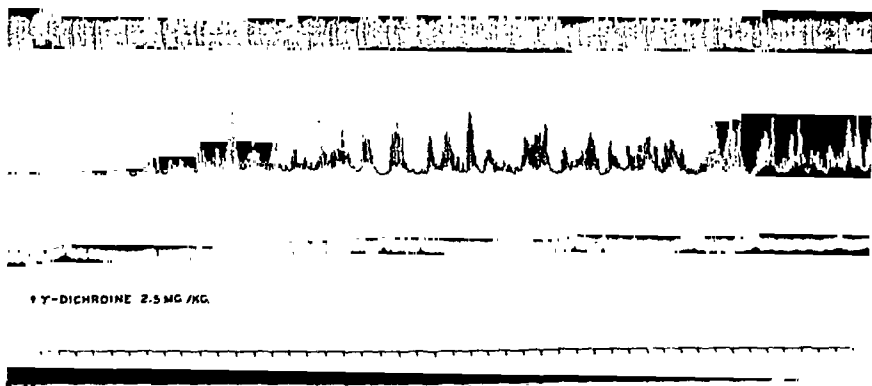


FIG. 2. ACTION OF γ -DICHRONE ON RESPIRATION, BLOOD PRESSURE, AND DUODENUM

Dog 6816, weighing 6.1 kgm., was anesthetized with 'Sodium Amytal', 70 mgm. per kgm. Tracings from top down are respiratory movements, duodenal movements, carotid blood pressure, time in minutes, and baseline. γ -Dichroine was injected intravenously.

and 5 by oral administration. When isolated strips of the rabbit's ileum were immersed in Locke-Ringer's solution, γ -dichroine inhibited the activity, as shown in figure 3. This action is entirely opposite to what may be expected as far as diarrhea is concerned. It is also not in agreement with the results in anesthetized dogs in which stimulation is the predominant feature of γ -dichroine. On account of the limited quantity of the alkaloid at our disposal, this phase of the study was not continued.

6. *Blood Sugar.* The effect of γ -dichroine on blood sugar was determined in albino rabbits. Fasting blood sugars were determined by the method of Hagedorn and Jensen (27), and the alkaloid was given by vein. Blood samples were taken after injection at 15 and 30 minutes, and 1, 2, 3, 4, 5, and 6 hours. Doses of 5 and 10 mgm. per kgm. in 4 rabbits gave very erratic blood sugar curves which can best be explained by the severe diarrhea produced, as mentioned above. A dose of 2.5 mgm. per kgm. in 3 additional rabbits caused a rise of

blood sugar, reaching a maximum in 3 to 4 hours. This dose was not followed by diarrhea. Figure 4 illustrates the blood sugar changes of these 3 animals.

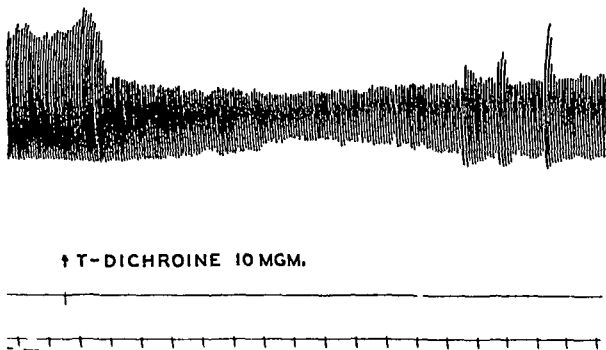


FIG. 3. ACTION OF γ -DICHROINE ON THE ISOLATED RABBIT'S ILEUM
The final concentration of γ -dichroine in the bath was 1:10,000. Note the inhibition of peristalsis which lasted more than 15 minutes

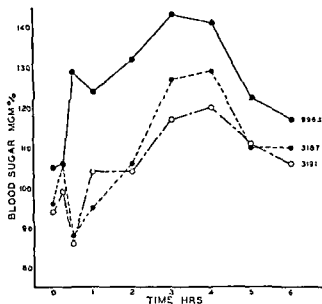


FIG. 4. ACTION OF γ -DICHROINE ON BLOOD SUGAR

The sexes and body weights of the 3 rabbits were as follows: No. 9963, female, 2.085 kgm.; No. 3187, female, 1.890 kgm., and No. 3191, female, 1.875 kgm. Each animal received γ -dichroine in the dose of 2.5 mgm. per kgm. intravenously.

7. *Antipyretic Action.* Previous investigation by Liu and his co-workers (14) revealed that the extract of Ch'ang Shan had an antipyretic action. It was

thus desirable to test the alkaloid for this action. The method of Smith and Hambourger (28) was employed. Briefly, albino rats were injected subcutaneously with a yeast suspension 18 hours prior to the test period. Rectal temperatures were taken by the use of a thermocouple. Acetylsalicylic acid was tested simultaneously and used as a standard for antipyretic activity. As shown in figure 5, γ -dichroine in a dose of 2.5 mgm. per kgm. orally in 5 rats has an antipyretic activity of greater magnitude and longer duration than acetylsalicylic acid. The dose of the latter was 25 mgm. per kgm., and the number of rats used was also 5.

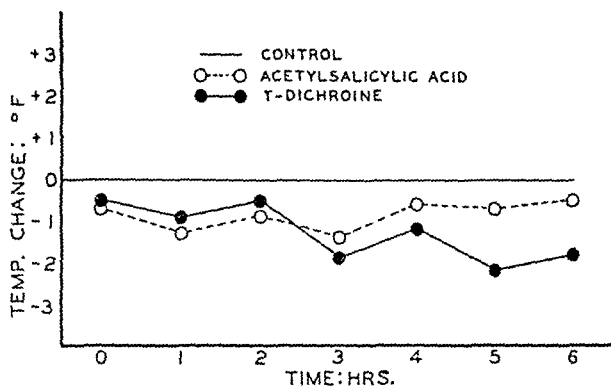


FIG. 5. THE ANTIPYRETIC ACTION OF γ -DICHROINE

Each curve represents the average differences of rectal temperature of a group of 5 rats from that of a control group of 5 rats, which occupies the straight line. All animals received a yeast suspension 18 hours prior to the temperature readings.

DISCUSSION. γ -Dichroine is unquestionably a potent antimalarial alkaloid in birds. It is also effective in monkey malaria, and, in all probability, is responsible for the activity of Ch'ang Shan or its extract in human malaria. These results substantiate the observations made by the Chinese many centuries ago. It is doubtful, however, that γ -dichroine can replace quinine, chloroquine, or other antimalarial agents, because it tends to cause nausea, vomiting, diarrhea, and hydropic degeneration of the liver in animals. Furthermore, it will be extremely costly to manufacture the alkaloid in large quantities from the root of the plant. A better plan is to synthesize and investigate the derivatives of quinazoline, since the latter is a degradation product of γ -dichroine (19).

Whether or not γ -dichroine is identical with febrifugine (21) and alkaloid II of Kuehl and his associates (22) will require further investigation. The Chinese and American chemists arrived at about the same composition by combustion analysis. There is a definite discrepancy, however, in their physical constants. All three groups obtained isomeric alkaloids of lower antimalarial potency. Direct comparison of the samples from the 3 laboratories is in order.

It is curious that γ -dichroine, in contrast with most other drugs, is roughly $3\frac{1}{2}$ times as toxic to mice orally as intravenously. No adequate explanation is on hand. Either elimination is extremely rapid by vein, or decomposition takes place in the gastrointestinal tract, and the degradation products are

especially toxic to the animal. In our laboratory, it was previously noted that the soluble derivatives of 4,4-diamino-1,1-diphenyl sulfone were also more toxic by mouth than by vein (unpublished data).

For want of material, several questions were left completely unanswered. The stimulating action of γ -dichroine on the dog's duodenum and the inhibiting action on the isolated rabbit's ileum need further exploration. Although the emetic action following intravenous injection is probably central in origin, it deserves more work to exclude the local action. It is also desirable to determine how the hyperglycemia is brought about.

SUMMARY

1. γ -Dichroine, an alkaloid of Ch'ang Shan, has a Q value of 148 against *Plasmodium lophurae* in ducklings, and a Q value of 137 against *P. relictum* in canaries. It is also active against *P. cynomolgi* in monkeys.

2. γ -Dichroine in a single dose is approximately $3\frac{1}{2}$ times as toxic to mice by mouth than by vein. Repeated administration of γ -dichroine in mice may result in hydropic degeneration of the liver.

3. γ -Dichroine induces vomiting in pigeons following intravenous injection, the $\text{EmD}_{50} \pm \text{S. E.}$ being 0.132 ± 0.04 mgm. per kgm.

4. γ -Dichroine has no effect on blood pressure and respiration in anesthetized dogs in the dose of 2.5 mgm. per kgm., but slightly lowers blood pressure with an acceleration of the respiratory rate in the dose of 4 mgm. per kgm.

5. γ -Dichroine stimulates duodenal peristalsis in anesthetized dogs, but inhibits the activity of the isolated rabbit's ileum. It causes diarrhea in non-anesthetized rabbits and rats following intravenous injection.

6. γ -Dichroine produces hyperglycemia in rabbits in the dose of 2.5 mgm. per kgm. when injected intravenously. In rats, the same dose when given by mouth shows a slightly greater antipyretic action than acetylsalicylic acid.

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REFERENCES

1. CHEN, K. K.: Fed. Proc., (in press).
2. LI, S. C.: Pentsao Kang Mu, chapter 17, part A, 1596.
3. CHEN, C. J.: Chinese Dispensatory, 2: 1143, Global Press, Shanghai, 1935.
4. STUART, G. A.: Chinese Materia Medica, 292, American Presbyterian Mission Press, Shanghai, 1911.
5. BOTANICAL NOMENCLATURE, 885, Commercial Press, Shanghai, 1918.
6. JANG, C. S.: Chin. Med. J., 62: 185, 1944.
7. YANG, S. T.: J. Am. Pharm. A., Scient. Sect., 37: 458, 1948.
8. YU, T. C.: J. Chin. Pharm. A., 7: 24, 1948.
9. KIMURA, K.: J. Pharm. Soc. Japan, 48: 884, 1928.
10. NAKAO, M.: J. Pharm. Soc. Japan, 48: 893, 1928.
11. TERADA, Y., SUZAKI, K., NAMURA, T., AND TADA, E.: Nagoya Igakkai Zasshi, 54: 43, 1941; through Chem. Abst., 42: 724, 1948.
12. KIMURA, K., AND SHIMADA, H.: J. Pharm. Soc. Japan, 48: 896, 1928.
13. TERASAKA, M.: J. Pharm. Soc. Japan, 51: 707, 1931.

14. LIU, S. K., CHANG, Y. T., CHUAN, T. K., AND TAN, S. C.: *Nat. Med. J. China*, 27: 327, 1941; *Chin. Med. J.*, 59: 575, 1941.
15. JANG, C. S., AND CHOU, T. C.: *Nat. Med. J. China*, 27: 137, 1943.
16. JANG, C. S., FU, F. Y., WANG, C. Y., HUANG, K. C., LU, G., AND CHOU, T. C.: *Science*, 103: 59, 1946.
17. JANG, C. S., FU, F. Y., HUANG, K. C., AND WANG, C. Y.: *Nature*, 161: 400, 1948.
18. CHOU, T. Q., JANG, C. S., FU, F. Y., KAO, Y. S., AND HUANG, K. C.: *Science (Chinese)*, 29: 49, 1947.
19. CHOU, T. Q., FU, F. Y., AND KAO, Y. S.: *J. Am. Chem. Soc.*, 70: 1765, 1948.
20. WISELOGLE, F. Y.: *A Survey of Antimalarial Drugs*, 2: 1603, 1946.
21. KOEPFLI, J. B., MEAD, J. F., AND BROCKMAN, J. A., JR.: *J. Am. Chem. Soc.*, 69: 1837, 1947.
22. KUEHL, F. A., JR., SPENCER, C. F., AND FOLKERS, K.: *J. Am. Chem. Soc.*, 70: 2091, 1948.
23. TONKIN, I. M., AND WORK, T. S.: *Nature*, 156: 630, 1945.
24. HOOPER, D.: *Nature*, 157: 106, 1946.
25. WISELOGLE, F. Y.: *A Survey of Antimalarial Drugs*, 1: 485, 1946.
26. SCHMIDT, L. H.: Private communication.
27. HAGEDORN, H. C., AND JENSEN, B. N.: *Biochem. Ztschr.*, 135: 46, 1923.
28. SMITH, P. K., AND HAMBOURGER, W. E.: *THIS JOURNAL*, 54: 346, 1935.

HEINZ BODY FORMATION BY CERTAIN CHEMICAL AGENTS

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INTRODUCTION. Heinz bodies, also known as Heinz-Ehrlich-, inner- or inclusion bodies, are refractile granules or particles sometimes found in erythrocytes.¹ These bodies were named after the pharmacologist, Robertz Heinz, who in 1890 (2) discovered them in the red blood cells of animals poisoned by certain aromatic compounds. In general, such formation was limited to phenylhydrazine and its derivatives, as well as to some aromatic amino and nitro compounds, although hydroxylamine and chlorates were found to have similar action. The work of Moeschlin and Hurschler (3-7) on the formation of Heinz bodies both *in vivo* and *in vitro* by certain sulfonamides caused renewed interest in the subject. Since the initial work of Heinz, more than a hundred investigations have been carried out in the attempt to elucidate the chemical nature of Heinz bodies, to account for their mechanism, and to devise methods for staining and differentiating them from similar particles.

During an investigation of the effect of stibine (antimony hydride, SbH_3) on experimental animals, the presence of small refractile granules was occasionally noted in the erythrocytes of these subjects. These granules were first observed by us in mice and guinea pigs and later in other species and it was initially assumed that they were Heinz bodies. In the course of work with other hemolytic and chemical substances similar inclusion bodies were noted. This research was undertaken in order to investigate their behavior and to establish their identity. Due to the exploratory nature of the study, fewer animals were used in testing each substance than would be necessary for statistical evaluation of the data. Since this work was incidental to the stibine study, to be reported elsewhere, further work is not contemplated at the present time.

METHODS OF OBSERVATIONS OF HEINZ BODIES. Using wet preparations of blood, the small, colorless refractile granules in the erythrocytes were stained blue with methyl violet dye, in the manner suggested by Heinz (2).

As used in this work, 2.93 grams of methyl violet dye (C.I. 680: dye content 88 per cent) is shaken with 100 cc. of 0.6 per cent sodium chloride solution, filtered and diluted with an equal volume of 0.85 per cent NaCl. This results in an approximately half-saturated solution of the dye in 0.73 per cent NaCl. In use, a drop of this solution is placed on a slide, and covered with a slip containing a small drop of blood on the bottom surface. After standing for 2-3 minutes the staining of any Heinz bodies present should be nearly complete. Observations under high power (1400-1500 \times) are best made on thinner fields where the red cells are sufficiently separated to enable counts to be carried out. Using critical illumination and by focusing up and down, blue particles as small as a fraction of a

¹ A review of the extensive literature on Heinz body phenomenon has been given elsewhere (1).

micron can be seen, the rolling of the cells during movement facilitating this observation. Using this technique, the occurrence of the so-called Rand bodies (8), which might be confused with Heinz bodies, was only rarely observed. Almost without exception the Heinz bodies were found only in mature red cells, the reticulocytes only rarely showing these particles.

Usually 8 or 10 fields were examined for the presence of Heinz bodies if few were seen. However, when these were numerous a count was made, usually 100 or 200 cells being carefully studied. The number of red blood cells having one or more blue particles was determined and a rating was made in accordance with the following scheme:

Percentage showing Heinz bodies	Evaluation
Occasional	\pm
1-10%	+
11-40%	2+
41-75%	3+
76-100%	4+

During the investigation of this phenomenon a new technique for staining these granules in smears was worked out (9). This involved treatment of the fresh air-dried smears with 0.2 per cent methyl violet in 95 per cent ethyl alcohol for one-half minute. It was found that preliminary fixation with methyl alcohol resulted in shedding of many of the particles from the erythrocytes and these could be seen on the slide. With the new technique the granules were fixed sufficiently to hold them within the cells while they were being stained. Some of the granules, however, were removed initially from the cells through trauma produced by the smearing operation. For these reasons the number of intraerythrocytic particles was much greater in the wet preparations than it was in the fixed slides. Hence the former method was preferred except for photographic purposes. By means of a special technique, using electronic photoflash equipment (10), photomicrography of Heinz bodies in wet preparations was found to be practicable.

EXPERIMENTAL PROCEDURES. A number of chemical substances were used experimentally in order to investigate the production of so-called inclusion bodies in the erythrocytes, several kinds of experiments being tried.

In the experiments made *in vivo*, stibine, a volatile substance, was administered to various species of animals in exposure chambers at predetermined concentration levels. Ordinarily a single one-hour exposure was used but with higher concentrations the time was often shortened. Sulfanilamide was given either by intraperitoneal injection of an aqueous solution, or of a suspension in gum acacia, or by using a 0.3 per cent solution of the material in the drinking water. Aqueous solutions of the remaining substances were ordinarily used for intraperitoneal injection, the dosage being calculated in terms of mgm./kgm. body weight.

The animals used in these experiments were drawn from the stock colony of the National Institutes of Health and only those in apparent good health were used. Care was taken to test the blood of the animals to be used experimentally and reject those showing more than an occasional inclusion body (\pm rating). The white mice used were of the N.I.H. strain, derived in turn from the white Swiss variety.

Finally, a few *in vitro* experiments were conducted using freshly shed mouse or guinea pig blood. One cc. of blood was mixed with 25 cc. of solution composed of 7 vol. of Locke's solution (11) to 3 vol. of water and containing the toxic substance in the desired concentration. After testing blood from tail or ear for Heinz bodies, the animal was bled and the blood was mixed immediately with the solution. The mixture was incubated at 37°C. and tests were made on the red cells at various intervals thereafter in the manner described.

ACTION OF STIBINE. Using wet blood preparations, it was observed that refractile granules appeared in mature erythrocytes of mice immediately follow-

ing a single one-hour exposure to stibine at a concentration level of 70 p.p.m. With a guinea pig given a similar exposure, the granules appeared after a few hours. Within six hours, 90 per cent and 75 per cent of the mouse and guinea pig cells, respectively, showed similar alterations.

Continuous observation over a period of 24 hours was carried out on 20 guinea pigs exposed to 53 p.p.m. of stibine for 1 hour. Hematological examinations were made immediately following exposure and every 4 hours thereafter. One animal died shortly after the end of the exposure. Of the remaining 19 animals, 4+ inner bodies were shown by 16 within 8 hours and by all within 12 hours. In 2 animals one or more tiny particles were present in small numbers within 4 hours. Blood destruction was evidenced not only by hemolysis and drop in red count but by hemoglobinuria in all but one animal. The inner bodies were present in the blood of all guinea pigs when they were sacrificed at the end of 3 days, varying in amounts from \pm (1 animal) to 4+ (7 animals). Growth in the size of the particles over the three-day period was quite evident. Similar results were noted in another series of 25 guinea pigs, having a single exposure to stibine, all of the animals showing the presence of intraerythrocytic particles when examined 24 hrs. after exposure. Fig. 1 shows the typical appearance of inner bodies in erythrocytes from a guinea pig 4 days after exposure of the animal to stibine.

The rat is apparently more resistant, for the bodies appeared later and were less numerous than in the other species. The granules were observed also in rabbits, cats, dogs, and monkeys.

The bodies were found to persist for relatively long periods of time. In mice they were observed for 55 days, in a rat for 33 days and in a guinea pig for 11 days following a single exposure to stibine. These granules stained well with methyl violet and gentian violet, both supravitaly and in smears, and they were insoluble in water, dilute acetic acid and ethyl and methyl alcohols. Their sizes varied with the species, the particles usually being much smaller in guinea pigs and rabbits than in mice. Furthermore, the particles had all the morphologic characteristics described by Heinz in his original communication (2). It seems evident, therefore, that the bodies produced by stibine are identical with the Heinz bodies described so many years ago.

Attempts were made to produce Heinz bodies *in vitro* by passing stibine through heparinized blood of various species of animals. Although morphologic changes were evidenced by the formation of spine cells with guinea pig blood, no Heinz bodies were observed in any case.

ACTION OF SULFANILAMIDE. Using the method of Figge (12), Heinz bodies were produced in mice given a 0.3 per cent solution of sulfanilamide for their drinking water. In less than 24 hours the presence of blue-staining refractile granules could be observed when using the supravital technique mentioned above. Fig. 2 shows the appearance of the Heinz bodies, using the electronic photoflash technique (10) to stop the motion of the cells in the supravital preparation. At first the particles appeared as tiny strands or dots, many of which exhibited Brownian motion, and even the smallest ones could be easily seen as

the cells rolled over and came into focus. These intraerythrocytic bodies were found to stain well with methyl violet and in both morphologic and chemical behavior they exhibited the properties of Heinz bodies.

It was characteristic that as these particles developed they became larger and more nearly round in shape and at times they appeared to protrude and were attached to the cell by a thick membrane. Only rarely were the Heinz bodies observed leaving the cells but apparently they do so, since the particles were seen outside the cells and they were easily extruded on smearing blood containing these granules. Fig. 3 illustrates this extrusion of Heinz bodies.

Intraperitoneal injection of sulfanilamide in mice at levels of 1000 mgm./kgm., comparable to that used in the above experiments, resulted in Heinz bodies in the erythrocytes 2-3 hrs. after injection, a + and 2+ response being observed in 2 animals after 24 hrs.

The chronic effect of sulfanilamide, with prolonged Heinz body formation, was studied by administering 0.3 per cent solution of this drug as drinking water to white mice for a period of 6 months. The experimental group consisted of 3 males, 2 additional males being used as controls. In the first month, during the cleaning of the nests, one of the male test animals was inadvertently replaced by a female. This resulted in pregnancy so that it was of interest to study the offspring. During the test period 6 litters, or a total of 35 mice, were born to this mother. All of the young appeared to be in good health and Heinz bodies were not present in the newly born mice during the suckling period. An experiment in which sulfanilamide crystals were implanted in a suckling and in a mature mouse resulted in finding Heinz bodies in the blood stream of the mature but not of the young animal. Intraperitoneal injection of another pair of mice with the same substance (1000 mgm./kgm.) resulted in a maximum response of + (1 per cent) and 2+ (16 per cent) for the suckling and the mature animal, respectively.

On supravital biweekly examinations, Heinz body estimations throughout the 183-day period were always 4+ on all of the treated mice, the controls being negative during the entire period. During the later stages the Heinz bodies were very large, exhibiting poor to moderate staining qualities. Numerous erythrocytes were seen to have more than one inclusion body.

Before sacrificing the animals at the end of the six-month (183-day) period blood was taken and urine was secured from the bladder of each animal. No hemoglobin was found in any urine sample. Blood from the female mouse appeared to be quite normal with the exception of finding 4+ Heinz bodies. The liver and kidneys of this animal were normal but the spleen was very black and large. The data for the male test group are summarized in table 1.

The organ: body weight ratios for livers and kidneys did not differ greatly in the treated and the control groups; the spleens of the treated animals, although having higher ratios than those for the control group, were below the average found for animals of this weight (13) and hence did not indicate enlargement.

The histopathologic examination revealed no significant changes in the hearts or lungs of the test animals. The spleens showed 2+ to 4+ iron, \pm iron

in an occasional convoluted tubule and + iron in the Kupffer cells of the liver of one animal.

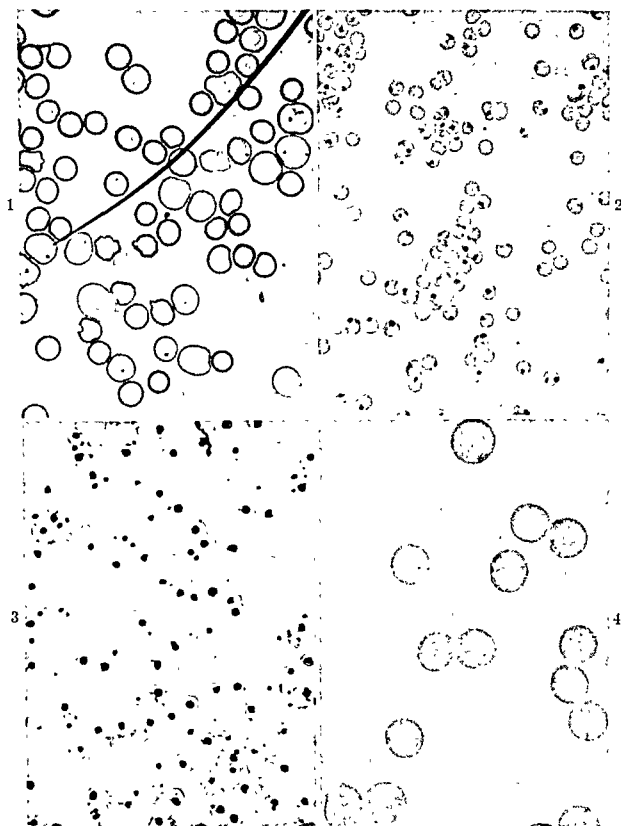


FIG. 1. HEINZ BODIES FORMED IN VIVO BY STIBINE. SMEAR OF GUINEA PIG BLOOD. $\times 660$

FIG. 2. HEINZ BODIES IN WET PREPARATION OF MOUSE BLOOD. $\times 660$

FIG. 3. EXTRUSION OF HEINZ BODIES. STAINED SMEAR OF MOUSE BLOOD. $\times 660$

FIG. 4. IN VITRO FORMATION OF HEINZ BODIES BY PHENYLHYDRAZINE. WET PREPARATION OF GUINEA PIG BLOOD. $\times 1350$

From the data given it will be noted that the prolonged ingestion of sulfanilamide had little influence on weight, red cell count, hemoglobin or hematocrit

the cells rolled over and came into focus. These intraerythrocytic bodies were found to stain well with methyl violet and in both morphologic and chemical behavior they exhibited the properties of Heinz bodies.

It was characteristic that as these particles developed they became larger and more nearly round in shape and at times they appeared to protrude and were attached to the cell by a thick membrane. Only rarely were the Heinz bodies observed leaving the cells but apparently they do so, since the particles were seen outside the cells and they were easily extruded on smearing blood containing these granules. Fig. 3 illustrates this extrusion of Heinz bodies.

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The histopathologic examination revealed no significant changes in the hearts or lungs of the test animals. The spleens showed 2+ to 4+ iron, \pm iron

however, a 4+ and a 2+ response were found with a guinea pig and a rabbit, respectively, in $\frac{1}{4}$ hr. after injection. Within 3 hrs. the rabbit also had a 4+ reaction. Other experiments with this same compound confirmed this finding that for a given dosage level fewer Heinz bodies were found in the rabbit than in the guinea pig.

At a dosage level of 50 mgm./kgm., acetylphenylhydrazine by intraperitoneal injection was found to produce 4+ Heinz bodies in mice within $\frac{1}{2}$ hour.

In vitro action of phenylhydrazine was found on mixing whole mouse blood with 7:3 Locke's solution containing 0.5 mgm. per cent of the drug, a few Heinz bodies being seen after 15 minutes, 2+ (35 per cent) after 2 hrs. and 4+ after 5 hrs. When the concentration of phenylhydrazine was increased to 10 mgm. per cent no Heinz bodies could be found, the drug appearing only to attack the erythrocytes and causing them to disintegrate.

Similar experiments with guinea pig blood showed that at 20 mgm. per cent of the drug fewer Heinz bodies were found than when the concentration was 1 mgm. per cent. The chief action in the former case appeared to be the formation of knobby cells and alteration of the cell membrane which took a deep stain with methyl violet. At the lower concentration + (4 per cent) Heinz bodies were found in 1 hr. and 4+ (87 per cent) in 6 hrs. Fig. 4 is a photomicrograph of this wet preparation taken at the 6 hr. interval. Since the cells were moving, only a few of the Heinz bodies are in focus.

It is evident from these experiments that phenylhydrazine and its acetyl derivative are very effective in forming Heinz bodies, and changes in the erythrocytes of the mouse and other species can be detected within a few minutes under suitable conditions.

ACTION OF OTHER CHEMICAL AGENTS. Several other chemical substances were investigated during this study, using white mice as experimental subjects, the preparations being given intraperitoneally in aqueous solution. The use of these compounds was suggested either because of their marked toxicity or their known hemolytic action. In a few cases inorganic oxidizing or reducing agents were tried.

Observations for Heinz bodies were made at intervals, the first usually being taken after $\frac{1}{4}$ hr. Ordinarily evaluations were repeated at 1-4 hr. intervals during the first day and then 24 hrs. later. However, with the very toxic materials at high dosage levels, some of the animals succumbed in a short time after injection, thus preventing further observations. The results are summarized in table 2, the maximum Heinz body rating and the corresponding time being indicated for each substance used.

It can be seen that in general the organic compounds were much more effective than the inorganic materials in inducing Heinz body formation. However, saponin, a powerful hemolytic agent, was much less effective than aniline. A number of the highly toxic inorganic compounds were almost without effect in producing intraerythrocytic particles although their lethal effects were quite evident. The reducing agents, sodium nitrite and sulfite and hydroxylamine, appeared to be superior to the oxidizing agents tried (chlorate, dichromate and

TABLE 2

Summary of Heinz body response of white mice following intraperitoneal injection of various substances

SUBSTANCE	DOSAGE	NO. OF ANIMALS	MAXIMUM HEINZ BODY EVALUATION	TIME OF OBSERVATION AFTER INJECTION
	<i>mgm./kgm.</i>			<i>hr.</i>
Aniline	40	2	2+	$\frac{1}{4}$
			\pm	1
Aniline	400	1	4+	20
Pyrogallol	200	2	+	$\frac{1}{4}$
			\pm	3
Saponin	50	1	Neg.	4
Saponin	100	2	\pm	1
			+	3
2-4 Toluylene diamine	100	2	+	3
			Neg.	3
Cobalt chloride	100	1	Neg.	6
Cobalt sulfate	100	1	Neg.	6
Ferrous sulfate	100	2	Neg.	23
			Neg.	23
Hydroxylamine hydrochloride	12.5	1	2+	1
	100	1	3+	$\frac{1}{4}$
Lead acetate	100	1	\pm	20
Lead acetate	400	1	\pm	20
Lead chloride	100	1	+	21
Mercuric chloride	5	1	Neg.	4
Mercuric chloride	10	1	+	4
Mercuric chloride	100	1	Neg.	$\frac{1}{4}$
Potassium chlorate	100	2	+ ?	1
			Neg.	20
Sodium dichromate	100	3	2+	1
			Neg.	3
			Neg.	4
Sodium nitrate	500	2	\pm	1
			Neg.	3
Sodium nitrite	100	1	\pm	$\frac{1}{2}$
Sodium nitrite	200	2	+	$\frac{1}{4}$
			Neg.	$\frac{1}{2}$
Tartar emetic	37.5	1	Neg.	96
	100	1	Neg.	$\frac{1}{2}$

nitrate) although ferrous sulfate was without effect. It is of interest to note that tartar emetic, though exhibiting marked toxicity at the high dosage levels, showed scarcely any effect on Heinz body formation, in marked contrast to the action of antimony in the form of the hydride. By far the greatest action for inorganic materials was exhibited by hydroxylamine which is both a nitrogen compound and a reducing agent.

In vitro action of hydroxylamine hydrochloride (1 mgm. per cent) was observed with mouse blood, a 2+ rating being found 11 minutes after mixing and a 3+ reaction after one-half hour. At a lower concentration of hydroxylamine (0.25 mgm. per cent) the action was slower, the Heinz body ratings being \pm in 15 min., + (10 per cent) after 1 hr., 2+ (38 per cent) after 3 hrs. and 4+ after 24 hrs. It was evident from these observations that the destructive action on the erythrocytes as well as the number and size of Heinz bodies was dependent on the concentration of the drug.

DISCUSSION. The mechanism of Heinz body formation and the site of their origin have not been elucidated. The wide variety of compounds, some of which are inorganic, capable of inducing intraerythrocytic changes, lends support to the view that Heinz body formation is due to partial destruction of some portion of the red blood cell, as a result of toxic action. The view that these bodies are formed in the peripheral circulation is supported by the fact that usually only mature cells and not reticulocytes have Heinz bodies and also by the fact that *in vitro* formation of such particles has been demonstrated. Although difficult to show photographically, the rapidity of this formation can easily be shown by visual observation using wet blood preparations. The more favorable conditions present in the living tissue are presumably responsible for the reaction *in vivo* usually being much greater than that *in vitro*. The persistence of Heinz bodies after a single dose of a substance is of great interest since it points to a relatively long life of some of the erythrocytes, amounting to 30-55 days in the mouse.

Although Heinz bodies were present in the majority of the erythrocytes of mice fed sulfanilamide during the 6-7 month test period, no hemolytic anemia was evident. This is in marked contrast to man, in which the presence of numerous Heinz bodies would be regarded as serious (3). Furthermore, the ability to form Heinz bodies is apparently not related either to the ability to form methemoglobin or to cause hemolysis. For example, saponin, which is regarded as a powerful hemolytic agent, was found to be relatively ineffective in inducing Heinz body formation in mice whereas sulfanilamide was able to produce these particles in nearly all erythrocytes in a short time. The most toxic substances appear to be those which are able to cause hemolysis, methemoglobin and Heinz bodies.

The production of Heinz bodies by stibine, a volatile hydride, is of interest since for many years it was believed that this phenomenon was almost exclusively confined to nitro compounds of the benzene series. Little interest appears to have been shown in studying the action of inorganic compounds in this field.

The fact that Heinz bodies were not found in suckling mice, the mother of which exhibited numerous intraerythrocytic particles, suggests some sort of a

protective mechanism in the young. It has been shown (14) that in pregnant rabbits treated with sulfanilamide this drug can pass from the maternal to the fetal circulation. Likewise transmission across the placental barrier has been demonstrated for humans (15). Moreover, transmission of this same drug through the milk of nursing mothers has been established (16). Consequently it was expected that the young mice would have Heinz bodies. However, the experiments on implantation and intraperitoneal injection confirmed the earlier observation and indicated a difference in susceptibility of young and mature mice to this drug.

The difficulty of finding a solution capable of maintaining red blood corpuscles of various species without marked morphologic changes during a supravital examination is well known. For this reason some differences in behavior of the Heinz bodies may be expected for various species. However, the great similarity in morphologic appearance, in their staining characteristics and in their physical and chemical behavior to the particles described earlier by Heinz, makes it quite evident that they should be designated as Heinz bodies.

SUMMARY AND CONCLUSIONS

The formation and properties of intraerythrocytic bodies produced by the action of stibine, phenylhydrazine hydrochloride, sulfanilamide and certain other chemical substances have been studied in experimental animals. Moreover, *in vitro* formation has been observed in mouse blood with phenylhydrazine hydrochloride, with sulfanilamide and with hydroxylamine hydrochloride. With respect to their various staining and morphologic characteristics, these particles have been found to conform to the properties of Heinz bodies.

Improved methods for staining Heinz bodies both in wet preparations and in smears have been worked out. Recognition and enumeration of these particles in their early stages have been facilitated by supravital examination of blood.

The formation of Heinz bodies by certain organic and inorganic compounds is dependent on a number of factors, among which are the chemical nature of the compound, dosage, route of administration and species differences. The Heinz bodies themselves differ in time of appearance, abundance, size and persistence.

Stibine and hydroxylamine hydrochloride are powerful inorganic Heinz body-producing substances while among the organic compounds, phenylhydrazine hydrochloride and acetylphenylhydrazine are very vigorous in their action. Sulfanilamide produces many Heinz bodies in mice and the lack of marked systemic effect, even after a period of months, makes this a useful substance for the study of this phenomenon.

Intraperitoneal injection of a toxic substance is a convenient and rapid method of determining its ability to form Heinz bodies. Because of the ease with which these bodies can be produced in mice, this species is a useful experimental animal. Guinea pigs and rabbits are less serviceable since their erythrocytes are more resistant than those of mice. Hemolytic activity and ability of a substance to form Heinz bodies are not uniformly related; nor is the persistence of these particles related to the maximum number found.

Further experimental work is needed in order to answer many of the remaining questions about the composition, site of origin and fate of Heinz bodies.

ACKNOWLEDGMENT. We wish to thank Drs. S. S. Spicer, B. L. Horecker and R. C. Dunn and Mrs. D. C. Peterson and Mr. E. C. Thompson for their aid in this investigation.

REFERENCES

1. WEBSTER, S. H.: Unpublished Mss, 1948. To appear in *Blood*.
2. HEINZ, R.: *Virch. Arch. f. path. Anat.*, **122**: 112, 1890.
3. MOESCHLIN, S.: *Schweiz. med. Wehnschr.*, **70**: 786, 1940.
4. MOESCHLIN, S.: *Schweiz. med. Wehnschr.*, **71**: 789, 1941.
5. MOESCHLIN, S.: *Folia haemat.*, **65**: 345, 1941.
6. MOESCHLIN, S., AND HURSCHLER, H.: *Schweiz. med. Wehnschr.*, **70**: 972, 1940.
7. HURSCHLER, H.: *Arch. f. exper. Path. u. Pharmacol.*, **197**: 438, 1941.
8. JÜRGENS, R., AND SCHÜRER, W.: *Schweiz. med. Wehnschr.*, **75**: 1055, 1945.
9. WEBSTER, S. H., LILJEGREN, E. J., AND ZIMMER, D. J.: *Stain Technol.*, **23**: 97, 1948.
10. WEBSTER, S. H., LILJEGREN, E. J., AND ZIMMER, D. J.: *J. Biol. Photographic A.*, **16**: 99, 1948.
11. LOCKE, F. S.: *Zentralbl. f. Physiol.*, **14**: 670, 1901.
12. FIGGE, F. H. J.: *Anat. Rec.*, **94**: 461, 1946.
13. WEBSTER, S. H., LILJEGREN, E. J., AND ZIMMER, D. J.: Unpublished work.
14. LEE, H. M., ANDERSON, R. C., AND CHEN, K. K.: *Proc. Soc. Exp. Biol. and Med.*, **38**: 366, 1938.
15. SPEERT, H.: *Bull. Johns Hopkins Hosp.*, **63**: 337, 1938.
16. HEPBURN, J. S., PAXSON, N. F., AND ROGERS, A. N.: *Arch. Pediat.*, **59**: 413, 1942.

THE ANTIFILARIAL ACTION OF CYANINE DYES¹

I. THE RELATIVE ANTIFILARIAL ACTIVITY OF A SERIES OF CYANINE DYES AGAINST *LITOMOSOIDES CARINII*, IN VITRO AND IN THE COTTON RAT

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The discovery, during a routine testing program, of the marked antifilarial activity of a large series of cyanine dyes when tested, *in vivo* and *in vitro*, against the filarial worm, *Litomosoides carinii*, of the cotton rat, has been reported in several preliminary publications from this laboratory (1, 2, 3). Unfortunately, this filaricidal effect of the cyanines was found to be highly selective. It was not demonstrable against several other filarial species and, in fact, early clinical trials against *Wuchereria bancrofti* have been unsuccessful. However, the experimental findings accumulated during the course of these studies were believed to be of sufficient fundamental importance to warrant their publication in detail.

In the coordinated program organized by the National Research Council, in 1944, for investigations of the chemotherapy of filariasis, several laboratories concentrated their efforts on the extension of existing knowledge of the antifilarial action of organic derivatives of antimony and arsenic (4, 7, 10, 11). In this laboratory, however, investigations were directed toward non-metallic compounds, in the hope of discovering a new approach to the chemotherapy of this disease.

A. THE ROUTINE TESTING OF COMPOUNDS FOR ANTIFILARIAL ACTIVITY. Since the two filarial parasites, *W. bancrofti* and *W. malayi*, which occur in the lymphatic system of infested human beings, have not been found in other animal species, it was necessary to conduct these studies with animals infested with a different, but morphologically related, filarial parasite. The timely report of Culbertson and Rose (4) on the chemotherapeutic action of organic antimonials against the filarial worm, *Litomosoides carinii*, in the Florida cotton rat, led to the selection of this same parasite and animal host for these studies. The occurrence of the worms in the pleural cavity made them readily accessible at autopsy for transfer in an intact condition to nutrient media. The size of the animals (under 200 grams) made them suitable for a large scale exploratory program and much to be preferred, for routine testing, to dogs infested with the heart-worm, *Dirofilaria immitis*. An adequate supply of naturally infested cotton rats was maintained by purchase from the Hegener Research Supply Company of Sarasota, Florida.

The "screening" procedure was based on the principle that each compound should be given in an amount approaching that maximally tolerated by the animal host. Schedules

¹ The work described in this series of papers was done, in part, under a contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Western Reserve University (August 1, 1944 to October 31, 1945); in part, under a contract between the Office of the Surgeon General, U. S. Army (November 1, 1945 to December 31, 1946); and in part, with the aid of a grant from the U. S. Public Health Service (since January 1, 1947).

involving frequent dosage were considered preferable, since they would favor the maintenance of a concentration of the drug in the tissue fluids bathing the parasites, and thus would increase the possibility of detecting minimal antifilarial activity in a compound, as a lead for further study. After a short period of experimentation with various schedules, it was decided, wherever possible, to administer compounds intraperitoneally every 8 hours for a total of 18 doses. For injection purposes the animals were driven from their individual wire-cloth cages into a tin and wire-cloth injector-tube which allowed handling and injecting with little danger of being bitten.² Autopsy was performed 40 hours after the final injection. The adult filariae were removed aseptically from the pleural cavities and placed in petri dishes containing 10 cc. of sterile nutrient medium³ for observation (the exact composition of the medium has been stated in the footnote); worms from an untreated rat were observed simultaneously. Characteristically, unaffected worms remained motile for at least 2 days when observed at room temperature. When motility was absent at the time of autopsy, and did not appear within 8 hours after the worms were removed from the rats, the filariae were considered dead.

The effect of drug treatment on the microfilariae, either *in vitro* or *in vivo*, was not studied, since it was known and was confirmed again in the course of these studies, that the susceptibility of these larvae to noxious agents may differ quite markedly from that of the parent worms, against which therapy was directed. Also, it was felt that routine "screening" of unrelated chemical substances for filaricidal activity *in vitro* would give information more likely to be misleading than helpful, since a great variety of substances were certain to be too toxic for filariae *in vitro*. However, extensive studies of the metabolism of *L. carinii* were conducted simultaneously by one of us (2, 3, 15), and sufficient information was obtained through this approach to facilitate progress materially when leads were obtained from the "screening" tests in animals. Under such conditions studies *in vitro* became of great importance.

B. THE DETECTION OF ANTIFILARIAL PROPERTIES IN THE CYANINE DYES. Among the many compounds studied, none was found to possess appreciable activity until a member of the group of compounds known as cyanine dyes was tested. This compound, (1-amy1-2,5-dimethyl-3-pyrrole) (1,6-dimethyl-2-quinoline) dimethincyanine chloride (Chemotherapy Center #348), whose structural formula is shown in table I, was completely curative in the maximally tolerated doses used. On further study it became evident that a very high degree of activity was present, since the intraperitoneal injection of 0.1 mgm./kgm., at 8-hour intervals for 18 doses, regularly killed all filarial worms in all treated animals. Though delayed fatalities occasionally occurred when individual doses of 1.8 mgm./kgm. were administered according to above treatment schedules, individual doses of 1.25 mgm./kgm. on this schedule were well tolerated. These findings indicated that the absolute margin of safety was remarkably high.⁴

² This apparatus was designed by J. T. Litchfield, Jr., who at the time was working with R. N. Bieter, H. N. Wright and their associates on a similar project involving other members of this series of compounds (8, 9).

³ The medium consisted of 1 part sterile horse serum, generously supplied by Sharp and Dohme, Inc., and 3 parts of a buffered glucose-salt solution which permitted optimal metabolic activity and motility, and had the following composition: 0.137 *M* NaCl, 0.0027 *M* KCl, 0.0003 *M* CaCl₂, 0.001 *M* MgCl₂, 0.06 *M* sodium phosphate buffer (pH: 7.6), 0.02 *M* glucose.

⁴ The term "absolute margin of safety" is used to signify a relation that might be expressed by the ratio $\frac{LD_{50}}{CD_{95}}$, or $\frac{LD_{0-10}}{CD_{90-100}}$, if indications are to be given of the occasional death of an animal on a dose that is usually non-lethal and the occasional failure of a dose to

With the known antifilarial compounds of antimony, such as 'Neostibosan' and 'Anthiomaline', it was found impossible to kill all worms in any single animal even at maximally tolerated doses on similar treatment schedules.

Quantitative studies of the effect of #348 on the number of microfilariae present in the peripheral blood were not made for reasons already given. However, qualitative examination of single drops of peripheral blood in cover glass preparations revealed no gross differences in the degree of microfilaremia present before, during, and at the completion of the period of treatment. Later, quantitative studies with another cyanine dye (#863) (table IV) revealed that a slow decrease in the microfilaria count did occur over a period of some months. This was believed to be due to the spontaneous death of the embryos and their failure to be replaced because of the much earlier death of the adult worms during the period of therapy (16).

C. THE ANTIFILARIAL ACTIVITY OF CYANINE DYE #348 IN VITRO. When filariae from untreated rats were placed in petri dishes containing 10 cc. of the nutrient medium referred to previously, in the presence of various concentrations of #348, motility was lost within one hour at a concentration of 1:100,000. At a concentration of 1:1,000,000, motility was reduced within 2 hours, and up to 50 per cent of the worms were rendered immobile within 7 hours; in the case of the remaining 50 per cent, however, some motility was present after 72 hours, when the motility of control worms began to decrease. At a concentration of 1:10,000,000, no effect on the worms was detectable. It is of interest that the lowest dose of #348, which consistently cured rats following repeated injections was 0.1 mgm./kgm., since, on the basis of rapid and equal distribution throughout the tissues and tissue fluids, this amount of compound could result in a maximal concentration not exceeding 1 part per 10,000,000, prior to the occurrence of degradation or excretion. This point will be discussed further in a subsequent communication (16).

On the basis of the concurrent studies of the metabolism of *L. carinii* by Bueding (2, 3, 15), it seemed of greater and more fundamental importance to determine the effect of #348 on the specific metabolic behavior of the parasite, than on a general phenomenon such as motility. Thus, it was found that the cyanines produced a marked inhibition of the oxygen consumption of the adult filariae. The oxidative metabolism of the worm was inhibited by the concentrations of #348 ranging from 1:25,000,000 to 1:6,000,000. This decrease in respiration was associated with a compensatory increase in glycolysis and a decrease in glycogen synthesis. Under anaerobic conditions, on the other hand, no effect on glycolysis was observed. Only with concentrations 1,000 to 2,000

cure that is usually curative. The statistically valid margin of safety or therapeutic index, $\frac{LD_{50}}{CD_{50}}$, though an experimentally much more reproducible value, often gives an impression of innocuousness that is not justified by the facts. Although many cyanines, administered either orally or intraperitoneally, have wide "absolute margins of safety", they are by no means as innocuous as the ratio $\frac{LD_{25}}{CD_{25}}$ would indicate.

times greater was the oxygen consumption of mammalian tissue slices or homogenates affected by these compounds, a fact which is reflected in the high margin of safety in the chemotherapeutic tests described earlier. Furthermore, worms removed from cotton rats treated with subcurative doses of #348 showed markedly depressed respiratory activity and an increase in aerobic glycolysis, as compared to worms removed from untreated rats. It seemed probable, therefore, that these drugs exert their chemotherapeutic effect through the inhibition of one or more enzyme systems concerned with oxidative metabolism. The metabolism of the microfilariae obtained from pleural washings was unaffected by the drug, a circumstance that may be attributed to a difference in the metabolic characteristics of the larvae from those of the adult parasites (2, 3, 15). This observation is in agreement with the finding that the drug caused no prompt reduction in the microfilaremia of the cotton rat.

D. COMPARATIVE STUDIES OF THE ANTIFILARIAL ACTIVITY OF CYANINE DYES. It seemed highly desirable to extend these studies to other cyanine dyes because of the high degree of curative activity, the high margin of safety, and the specific antimetabolic effect of the one compound tested. Furthermore, Bieter, Wright and their associates had simultaneously disclosed antifilarial properties in a related series of styrylquinoline dyes (8, 9).

Fortunately for the chemotherapeutic program, a large number of these compounds had been synthesized by Dr. L. G. S. Brooker and his associates of the Eastman Kodak Company for other purposes (12, 13). Samples of these were generously submitted to the Committee on Medical Research of the National Research Council through Parke, Davis and Company, for study in this laboratory.⁵

Two procedures were used in screening these compounds for antifilarial activity against *L. carinii*. The first of these involved a comparison of the ability of the various cyanines to inhibit the oxygen uptake of the parasites. The second consisted of an assay, in cotton rats, of the chemotherapeutic and toxic properties of each compound.

1. *Assay, in vitro, of the antifilarial activity of cyanine dyes.* Adult filariae (15 to 20 mgm.) were transferred to small Warburg vessels (volume, 4 to 5 cc.) which contained 0.7 cc. of buffered glucose-salt solution.³ A small roll of filter paper, soaked with 0.1 cc. of 40 per cent KOH was placed in the center cup of each vessel. The oxygen uptake of the worms was measured over a period of 3½ hours at 38°C. in an atmosphere of air, in the conventional Warburg apparatus. During the initial period of 30 minutes, a slight increase in the respiration of the worms frequently occurred. After this time, the rate of oxygen uptake of the filariae remained constant over a period of at least three to four hours. The control respiration was recorded for the next 90 minutes, i.e., 30 to 120 minutes after the beginning of the experiment. Following this control period, 0.1 cc. of the glucose medium containing a cyanine dye was tipped from the side-arm into the main compartment of the vessel and the respiration was recorded for an additional 90 minutes. Addition of 0.1 cc. of the same

⁵ From the beginning of the study of this group of compounds, we have enjoyed the finest cooperation from Dr. Brooker and from Parke, Davis Laboratories. Further, this investigation has been facilitated in innumerable ways by Dr. Lucille Farquhar, technical aide of the National Research Council, who coordinated studies in this and related fields.

medium containing no cyanine dye did not affect the rate of oxygen uptake of the worms. All solutions of compounds to be tested were prepared immediately before the experiment. The antifilarial activity of each compound, *in vitro*, designated in the tables as the "in vitro index", was established in the following manner. The molar concentration of compound #348, which was used as a standard of reference, required to inhibit the respiration of the worms by 50 per cent (usually $2.6 \times 10^{-7} M$ or 1:10,000,000) was divided by the molar concentration producing a similar decrease in respiration in the case of the compound under assay.

2. *Assay, in vivo, of the antifilarial action of cyanines.* Further studies on the antifilarial action of #348 in cotton rats indicated that cures could be produced when dosage intervals of much greater than 8 hours were used. The minimal curative dose, when the drug was administered intraperitoneally at 8-hour intervals for 6 days, was 0.1 mgm./kgm.; a total dose of 1.8 mgm./kgm. When one injection was given daily for 5 days, an increase in the individual dose to only 0.2 mgm./kgm. was required; a total dose of 1.0 mgm./kgm. Furthermore, when the interval between the last dose and the time of autopsy was extended to 4 days, 0.3 mgm./kgm. daily for 3 days was required. When a single dose was given, followed by a period of one week, prior to autopsy, it was found that 1.35 mgm./kgm. would effect a cure. Later, the studies with other cyanines yielded similar results.

Accordingly, in "screening" various related compounds for their chemotherapeutic activity against *L. carinii* in the cotton rat, a dosage schedule was selected which involved one injection daily for 5 days. Autopsies, involving removal of worms from the pleural cavity for the purpose of observing the effect of therapy on motility, as described earlier, were performed 48 hours after the last dose. The following dose levels, expressed as the amount administered daily in mgm./kgm. of body weight, were used: 0.05, 0.10, 0.15, 0.20, 0.30, 0.40, 0.80, 1.6 or 2.0, 3.0, 4.0, 6.0, 8.0, 12.0, 16.0, etc. It was not necessary to use all these dose levels for each compound, and the data for any one compound were not obtained in a single test. Rather, an orientation test was performed first by treating several infested rats at one of the lower dose levels, and several uninfested rats at one of the higher dose levels. On the basis of the results obtained, higher or lower doses were then used in further experiments on additional rats to determine minimally curative and maximally tolerated doses. In this preliminary "screening" program, designed to separate compounds of high chemotherapeutic activity worthy of more extensive investigation, from those of low activity, only three animals were used per dose level of drug. The large number of compounds to be studied, the high cost of the infected animals, and the special handling which these separately housed animals required during maintenance, and especially during injection, made it necessary to set such a low figure for the number of animals to be used per drug. The values determined for each compound were: first, the minimum dose required to produce death of all worms in the three rats of a dosage group (minimum curative dose); and, second, the maximum dose allowing survival of all three rats in a dosage group (maximum tolerated dose). The margin of safety, or therapeutic index, was calculated by dividing the second value by the first. Such a therapeutic index was selected in preference to one involving 50 per cent end points, not only because of the small number of animals used, but also, as has been mentioned previously, because values representing complete curative responses, and complete survival, seemed more comparable to a clinical situation. Because both the toxicity and curative activity of these compounds increase rather slowly with increasing dosage, the statistical therapeutic index, based on 50 per cent responses, would have yielded figures of considerably higher magnitude.

In the data recorded in the following tables, the toxicity tests were performed with uninfested cotton rats of a single strain⁶. Earlier toxicity tests with a few cyanines, including #348, were performed with uninfested rats obtained from the same natural habitat as the infected ones. These last-mentioned rats were considerably more resistant to the lethal effects of the cyanines, than were those of the laboratory strain; hence, con-

⁶ Obtained from Tumblebrook Farms, Brant Lake, New York.

siderably higher therapeutic indices resulted from their use. Since our interest was in a comparison of the relative toxicity of these compounds so far as these lower animals were concerned, rather than in absolute values, we did not revert to the use of Florida rats for toxicity studies when this discrepancy became apparent. Rather, we repeated the earlier toxicity tests, on the daily injection schedules, with the laboratory strain of rats which were used thereafter.

In the case of some of the compounds presented in the tables, the "*in vitro* index" alone was determined. In almost all such cases, the amount of sample available was insufficient to permit studies *in vivo*. The small samples sufficed for the metabolic studies, and yielded additional information with regard to the relation of chemical structure to antifilarial activity. In a few other cases assays were performed only *in vitro* with compounds, other than cyanines, which were well-known from work in this and in other laboratories to be devoid of any chemotherapeutic effect *in vivo*. In this way it was possible to determine the presence or absence of a low degree of antifilarial activity, the existence of which was suggested by a remote chemical similarity to the cyanines. The results of the "screening" tests are presented in tables I to X.

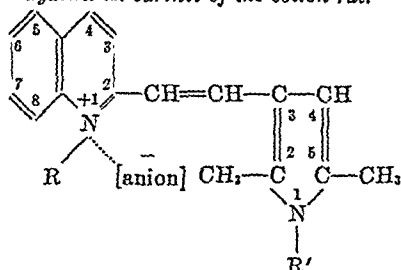
E. THE RELATION OF THE CHEMICAL STRUCTURE OF THE CYANINE DYES TO THEIR ANTIFILARIAL ACTIVITY AGAINST *L. CARINII*. It has been postulated earlier that the cyanines exert their chemotherapeutic effect through the inhibition of one or more enzyme systems concerned with the oxidative metabolism of the parasite. Further evidence for this, presented in the tables, is shown by the fact that every compound found to be active *in vivo* also inhibited filarial respiration *in vitro*. The fact that the reverse correlation did not exist, in other words, all compounds active *in vitro* were not active *in vivo*, is not a refutation of this postulate. In the complex animal organism many factors, such as rate and extent of excretion, and metabolic alteration, as well as unfavorable distribution, indubitably influenced the extent to which the inherent antifilarial activity of various compounds was able to become manifest. Thus, a discussion of the relation between chemical structure and the inherent antifilarial activity of these compounds would be more valid when based on observations made *in vitro*, in the absence of the modifying factors imposed by an animal host. Such a discussion is presented below, with supplementary remarks on the relation between chemical structure and "net" antifilarial activity; i.e., activity *in vivo*. Accordingly, the term "activity", as used in the following discussion, will refer to antifilarial activity *in vitro*, unless otherwise stated. The various related members of the cyanine series made available to us were prepared largely for purposes other than the chemotherapeutic program (12, 13). In a number of cases, however, the group led by Dr. Brooker kindly prepared new compounds, the structure of which was suggested by the antifilarial studies.

As shown in the tables, many different types of cyanines were found to possess antifilarial activity, and the synthesis of numerous derivatives of every structural type was impractical, at least until some knowledge was forthcoming in regard to the effectiveness of the cyanines against *W. bancrofti* in man.

1. (3-Pyrrole)(2-quinoline) dimethincyanines (table I). The most complete study of the effect of various substituent groups on antifilarial activity, both *in vitro* and *in vivo*, was made with this group of compounds which included #348, the cyanine originally found to be active against *L. carinii*. In compounds

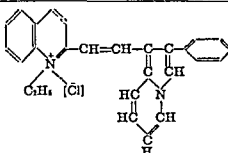
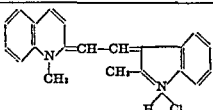
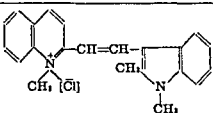
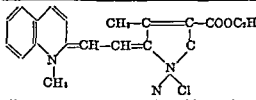
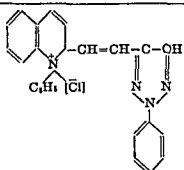
TABLE I

The relative antifilarial activity of a number of (3-pyrrole)(2-quinoline) dimethineyanines against *L. carinii* of the cotton rat.



CHEMOTHERAPY CENTER NO.	SUBSTITUENT GROUP						MINI- MUM CURA- TIVE DOSE	MAXI- MUM TOLER- ATED DOSE	THERA- PEUTIC INDEX	IN VITRO INDEX
	1		R'	6	7	8				
	R	anion								
348	CH ₃	Cl	C ₆ H ₁₁	CH ₃			0.2	2.0	1.0	*
804	CH ₃	Cl	CH ₃	CH ₃	—	—	>16.0	16.0	<1.0	0.5
711	CH ₃	Cl	C ₂ H ₅	CH ₃	—	—	8.0	8.0	1.0	1.5
999	CH ₃	Cl	C ₂ H ₅ (n)	CH ₃	—	—	2.0	4.0	2.0	1.5
1093	CH ₃	Cl	C ₂ H ₅ (iso)	CH ₃	—	—	1.6	4.0	2.5	1.0
998	CH ₃	Cl	C ₄ H ₉	CH ₃	—	—	2.0	2.0	1.0	1.25
348	CH ₃	Cl	C ₆ H ₁₁	CH ₃	—	—	0.2	2.0	10.0	1.0
802	CH ₃	Cl	C ₇ H ₁₅	CH ₃	—	—	0.2	1.0	5.0	0.4
1092	CH ₃	Cl	C ₁₀ H ₂₁	CH ₃	—	—	0.2	1.0	5.0	0.5
712	CH ₃	Cl	C ₁₂ H ₂₅	CH ₃	—	—	0.4	<4.0	<10.0	0.1
997	CH ₃	Cl	C ₆ H ₁₁ (cyclo)	CH ₃	—	—	0.4	2.0	5.0	1.5
803	CH ₃	Cl	C ₁₀ H ₁₉	CH ₃	—	—	—	—	—	0.002
799	CH ₃	CH ₃ C ₆ H ₄ SO ₄	C ₂ H ₅	CH ₃	—	—	—	—	—	0.8
713	CH ₃	Cl	C ₆ H ₅	OCH ₃	—	—	0.8	8.0	10.0	0.25
818	CH ₃	Cl	C ₆ H ₅	C ₆ H ₅	—	—	—	—	—	0.30
714	CH ₃	Cl	C ₆ H ₄ OC ₂ H ₅	CH ₃	—	—	0.3	<3.0	<10.0	0.6
715	CH ₃	Cl	C ₆ H ₅	N(CH ₃) ₂	—	—	1.6	6.0	4.0	0.7
800	CH ₃	Cl	C ₂ H ₅	—	—	—	>0.8	<3.0	<4.0	0.7
808	CH ₃	Cl	C ₆ H ₅	—	—	CH ₃	—	—	—	1.0
803	CH ₃	Cl	C ₆ H ₅	—	—	OCH ₃	0.8	2.0	2.5	0.7
819	CH ₃	Cl	C ₆ H ₅	—	—	C ₆ H ₅	—	—	—	0.25
807	CH ₃	Cl	C ₆ H ₄ Cl	CH ₃	—	—	0.8	<4.0	<5.0	0.6
815	CH ₃	Cl	C ₆ H ₅	NH·CO·C ₆ H ₁₁	—	—	>2.0	2.0	<1.0	0.05
811	CH ₃	Cl	C ₆ H ₅	Cl	—	—	>6.0	7.5	1.0 or	0.4
									<1.0	
812	CH ₃	Cl	C ₆ H ₅	—	—	Cl	>4.0	4.0	<1.0	1.0
816	CH ₃	Cl	C ₆ H ₅	—	Cl	—	1.6	4.0	2.5	0.3
962	CH ₃	Cl	C ₆ H ₁₁	—	Cl	—	>1.6	4.0	<2.5	0.7
814	CH ₃	Cl	C ₆ H ₅	—	CH ₃	—	>4.0	4.0	<1.0	1.0
817	CH ₃	Cl	C ₆ H ₅	C ₆ H ₁₁ (tert)	—	—	>1.6	<1.6	<1.0	0.4
959	H	Cl	C ₆ H ₅	CH ₃	—	—	>128.0	128.0	<1.0	0
963	C ₂ H ₅	Cl	C ₆ H ₁₁	CH ₃	—	—	0.3	4.0	13.0	1.5
349	C ₂ H ₅ OH	Cl	C ₆ H ₁₁	CH ₃	—	—	3.2	8.0	2.5	0.5
964	C ₆ H ₇	Cl	C ₆ H ₁₁	CH ₃	—	—	0.15	2.0	13.0	0.3
965	C ₆ H ₁₁	Cl	C ₆ H ₁₁	CH ₃	—	—	0.15	2.0	13.0	0.5
943	CH ₃	Cl	C ₆ H ₁₁	—	—	—	0.40	4.0	10.0	1.0
967	CH ₃	Cl	C ₆ H ₁₁	OCH ₃	—	—	0.40	6.0	15.0	1.0
805	CH ₃	Cl	CH ₃ CH ₂ OCH ₃	CH ₃	—	—	>2.0	2.0	<1.0	0.5

TABLE I—Continued

CHEMOTHERAPY CENTER NO.	SUBSTITUENT GROUP						MINI- MUM CURA- TIVE DOSE	MAXI- MUM TOLER- ATED DOSE	THERA- PEUTIC INDEX	IN VITRO INDEX
	1		R'	6	7	8				
	R	anion								
801	C ₂ H ₅	Cl	C ₆ H ₅	CH ₃	—	—	—	—	—	0.8
809	C ₇ H ₁₅	Cl	C ₆ H ₅	CH ₃	—	—	0.8	2.0	2.5	0.6
810	C ₁₁ H ₂₃	Cl	C ₆ H ₅	CH ₃	—	—	>4.0	4.0	<1.0	>0.1
813	CH ₃ CH ₂ OC ₂ H ₅	Cl	C ₆ H ₅	CH ₃	—	—	0.8	2.0	2.5	0.6
820	C ₆ H ₅	Cl	C ₆ H ₅	—	—	—	1.6	3.2	2.0	0.8
1276	CH ₃	Cl	CH ₂ C ₆ H ₄	CH ₃	—	—	0.8	2.0	2.5	1.0
1275							—	—	—	0.7
797							>16.0	16.0	<1.0	0.005
798							0.8	8.0	10	0.5
821	 a (2-pyrrole) (2-quinoline) dimethincyanine						—	—	—	<0.02
1278	 a (3-triazole) (2-quinoline) dimethincyanine						>16.0	16.0	<1.0	0.05

* Standard of reference (1.0).

having a methyl group in position -1 or -6 of the quinoline ring, the highest degree of activity was observed when an ethyl (#711), propyl (#999), or a cyclohexyl (#997) radical was attached to the pyrrole-N. A somewhat lower degree of activity was present when a methyl (#804), butyl (#998), or amyl (#348) radical was the substituent. A further lengthening of the side-chain produced a progressive decrease in activity (#802, #1092, #712, #805). Activity *in vivo*, on the other hand, was poor when the alkyl substituent on the pyrrole-N was short, and increased gradually to a maximum at C₆ through C₁₀, beyond which it could not be tested with accuracy due to the low degree of solubility, and of absorbability from the site of injection.

It is regrettable that the series of analogues discussed in the above paragraph, i.e., those having methyl groups in position -1 and -6 of the quinoline ring, with various substituents on the pyrrole-N, did not include the compound in which a phenyl group was the variant. The comparative effect of a phenyl group versus *one* alkyl radical, namely, the amyl, can be seen, however, in *other* compounds of the (3-pyrrole)(2-quinoline) series of table I, where substituents in other positions were the same. Thus, in each of the following four pairs of compounds the amyl radical conferred greater activity on the compound than did the phenyl: #713 and #967; #800 and #943, #962 and #816; #801 and #963. For the first two pairs of compounds this was also true *in vivo*, while for the third pair the phenyl compound was more active in cotton rats; a comparison was not made with the fourth pair.

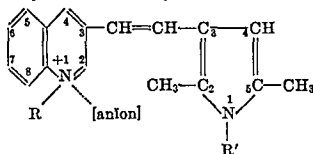
Data are also available in table I for a study of the effect on antifilarial activity of various substituents at the quaternary quinoline-N. With an amyl group on the pyrrole-N, replacement of a methyl group (#348) by an ethyl group (#963) at the quinoline-N produced an increase in activity, while further lengthening of the substituent alkyl chain led to decreased activity (#964 and #965). In this case, the changes in activity *in vivo*, though not marked, followed the reverse order. Replacement of the ethyl group of #963 by a β -hydroxyethyl group (#349) produced a marked decrease in activity both *in vitro* and *in vivo*. The deleterious effect of a long alkyl chain on the quinoline-N was also seen in comparing the relative activities of compounds whose pyrrole-N carried a phenyl radical, though it was outstanding only in going from a heptyl to an undecyl substituent (#801, #809, #810).

The effect of substituents at other positions than the nitrogens can be seen in various compounds with the common feature of a methyl radical on the quinoline-N and a phenyl on the pyrrole-N. When #800, which had no additional substituent, was altered by exchanging the hydrogen at C-8 for a methyl group (#808), or a chlorine atom (#812), a slight increase in activity resulted. When the methyl group was in position-6 (#714), instead of in position-8 (#808), a reduction in activity occurred. Similarly, decreased activity was observed with a chlorine atom in position-6 (#811) or -7 (#816), instead of in position-8 (#812) of the quinoline ring. A methoxy group in position-8 (#803) had no effect on activity, but a considerable decrease in activity occurred with a methoxy group in position-6 (#713). A phenyl group in position-6 (#819 or -8 (#818) resulted

in a decrease of activity of the same magnitude. Thus, compounds possessing $-\text{Cl}$ (#812), $-\text{CH}_3$ (#808), or $-\text{OCH}_3$ (#803) in position-8 of the quinoline ring appeared to possess higher activity than those in which a similar substituent was introduced in position-6 or -7. On the other hand, when no substituent was present in the -6, -7 or -8 position of the quinoline ring, as in #943, the antifilarial activity *in vitro*, remained equal to that of #348, which carried a methyl group at position-6. In the cotton rat the former compound was less active than the latter, but a corresponding decrease in toxicity permitted the therapeutic index to remain unchanged.

TABLE II

The relative antifilarial activity of a number of (3-pyrrole)(4-quinoline) dimethincyanines against *L. carinii* of the cotton rat



CHEMO- THERAPY CENTER NO.	SUBSTITUENT GROUP				MINIMUM CURATIVE DOSE	MAXIMUM TOLERATED DOSE	THERA- PEUTIC INDEX	IN VITRO INDEX
	1		R'	6				
	R	anion						
823	CH ₃	Cl	C ₆ H ₅	—	—	—	—	0.3
824	CH ₃	Cl	C ₆ H ₅	OCH ₃	1.6	24.0	15.0	0.25
955	CH ₃	Cl	C ₆ H ₁₁	—	0.8	2.0	2.5	0.8
1095	C ₂ H ₅	Cl	C ₆ H ₁₁	—	0.4	2.0	5.0	1.0
1096	C ₃ H ₇	Cl	C ₆ H ₁₁	—	0.2	1.0	5.0	0.7
1097	CH ₂ —CH ₂ OH	Br	C ₆ H ₁₁	—	>1.6	16.0	<10.0	0.3
1277	C ₄ H ₉	Cl	C ₁₀ H ₂₁	—	0.8	4.0	5.0	0.15

2. (3-Pyrrole)(4-quinoline) dimethincyanines (table II). As in the first group of compounds, greater activity was observed here when an amyl radical was attached to the pyrrole-N than when a phenyl was present (#955 and #823). Alteration of #823, by insertion of a methoxy group in position-6 of the quinoline moiety, did not significantly alter activity. An increase in the length of the alkyl radical on the quinoline-N augmented activity when the new substituent was an ethyl radical (#1095), but decreased it slightly with a propyl group (#1096). As in the previous group of cyanines, a β -hydroxyethyl group very significantly decreased activity. The detrimental effect, on activity, of an excessively long alkyl chain on the pyrrole-N is demonstrated again by comparison of the "in vitro index" of #1095 (amyl) and #1277 (decyl).

In regard to the effect of moving the attachment of the cyanine bridge from

TABLE III
The relative antifilarial activity of a number of cyanines of the diquinoline type against *L. carinii* of the cotton rat

CHEMO- THER- APY CENTER NO.	STRUCTURE	MINI- MUM CURA- TIVE DOSE	MAXI- MUM TOLER- ATED DOSE	THERA- PEUTIC INDEX	IN VITRO INDEX
Dimethincyanines					
834		mgm./ kgm. >3.2	mgm./ kgm. 6.0	<2.0	2.0
837		>4.0	<8.0	<2.0	1.0
Monomethincyanines					
853		—	—	—	0
856		—	—	—	0.3
		—	—	—	0.004

TABLE III.—Continued

CHEMO- THER- APY CENTER NO.	STRUCTURE	MINI- MUM CURA- TIVE DOSE	MAXI- MUM TOLER- ATED DOSE	THERA- PEUTIC INDEX	IN VITRO INDEX
<i>Monomethincyanines—Continued</i>					
858		>4.0	4.0	<1.0	0.7
865		—	—	—	0.5
892		3.2	12.0	4.0	1.0
947		>2.4	<4.0	<2.0	1.0
855		—	—	—	0

TABLE III—Continued

CHEMO- THER- APY CENTER NO.	STRUCTURE	Monomethincyanines—Continued			
		MINI- MUM CURA- TIVE DOSE	MAXI- MUM TOLER- ATED DOSE	THERA- PEUTIC INDEX	IN VITRO INDEX
857		mgm./ kgm. >4.0	mgm./ kgm. >6.0	<1.5	0.5
866		0.8	2.0	2.5	0.1
Trimethincyanines					
871		0.15	2.0	13.0	0.3
89		>0.4	<0.8	<2.0	0.25
		4.0	<6.0	<1.5	0.15

TABLE III—*Concluded*

CHEMO- THER- APY CENTER NO.	STRUCTURE	MINI- MUM CURA- TIVE DOSE	MAXI- MUM TOLE- RATED DOSE	THERA- PEUTIC INDEX	IN VITRO INDEX
<i>Trimethincyanines—Concluded</i>					
884		—	—	—	0.25
1000		—	—	—	0.15

position-2 to position-4 of the quinoline ring, no difference was observed in the activity of #713 (table I) and #824 (table II). The decrease in the activity of #824 *in vivo* was compensated for by its lower degree of toxicity, so that a slightly higher therapeutic index resulted. In several other instances where the cyanine bridge was moved to the new position, a decrease in activity *in vitro* was observed (#800 and #943 of table I versus #823 and #955, respectively, of table II). Numbers 943 and 955 both were tested chemotherapeutically and a similar difference in their potency was observed *in vivo*.

3. *Diquinoline cyanines* (table III). One striking factor involved in the relation of structure to pharmacological action of the cyanines was demonstrated in this group; this pertains to the nature of the connecting bridge between the two nitrogens. The three compounds in which both nitrogens were tertiary (#853, #854 and #855) showed no appreciable activity. The conversion of one nitrogen to the quaternary form resulted in the establishment of a resonating system of alternating double and single bonds in the chain connecting the two nitrogens. This was associated with a marked degree of antifilarial activity that was sustained despite many minor modifications in structure (table III).

The effect of a shift in the cyanine bridge from the 2-2' position to the 2-4' position, as shown in #856 and #857, was a moderate increase in activity.

No difference in antifilarial activity *in vitro* was present between the monomethine cyanine, #856, and the corresponding trimethine derivative. A similar comparison of #858 and #869, however, showed the monomethine compound to be considerably more active, since the addition of methyl groups in the 6-positions of the two quinoline rings increased appreciably the activity of the monomethine, but not that of the trimethine compound. A comparison of this sort was also made with two compounds of the dipyrrole type (#1113, #1114);

here the monomethine compound was very much more active than its trimethine analogue.

Few compounds were available in which alkyl radicals other than methyl were attached to the nitrogens in this series. The change from a methyl to an ethyl substituent was studied in the case of #856 and #947. It resulted in a threefold increase in activity.

The two dimethine compounds in this series were not analogous to any of the dimethincyanines of the pyrrole-quinoline type (tables I and II), hence no comparisons of activity could be made in this case.

A number of the compounds in this series showed a high degree of antifilarial activity *in vitro*. In fact, one of these, #834, had an "*in vitro* index" of 2.0, and, though several other cyanines equaled this potency, none of them exceeded it. Despite this, #834 did not have a high degree of antifilarial activity in the cotton rat. In fact, the only diquinoline compound which was highly active from the chemotherapeutic standpoint was #871. The "*in vitro* index" of this compound was not outstanding (0.3); hence this series affords a good example of the fact that the animal host may influence profoundly the antifilarial activity of the cyanines. It is interesting also that Bieter, Wright and their associates (8, 9) have reported a 2-2' trimethincyanine of the diquinoline type to have a high degree of antifilarial activity and a high therapeutic index in infested cotton rats; both nitrogens in this compound carried β -ethoxyethyl groups.

4. Two compounds which deserve special mention are #863 and #835 (table IV), whose structures are unique in that the second heterocyclic ring in each case was not present in any other of the compounds studied; each had an "*in vitro* index" of 2.0. In both cases the chemotherapeutic activity in infested cotton rats was not of a maximal order, but because their toxicity also was less than that of many other cyanines, the high therapeutic index which resulted made them members of the group of compounds selected for further study.

5. The pyrrole-pyridine cyanines depicted in table V were not outstanding in their activity, but they merit attention because of the deleterious effect produced by replacement of an amyl group on the pyrrole-N by a phenyl radical. This decrease was of a much greater magnitude than that resulting from a similar change of substituents in the pyrrole-quinoline series (table I). It may be pointed out also that the pyrrole-pyridine compound, #957, was half as active as its corresponding pyrrole-quinoline analogue (#943, table I).

6. A number of pyrrole-benzimidazole and pyrrole-benzothiazole cyanines were available for study (tables VI and VIII), and in a few cases "*in vitro* indices" of 1.0 were obtained. However, this high degree of activity did not carry over to the chemotherapeutic assays. A few pyrrole-benzoxazole (table VII) and pyrrole-benzoselenazole (table VIII) compounds were also studied, but none was outstandingly active, either *in vivo* or *in vitro*. In these cyanine types, replacement of an amyl by a phenyl radical was not detrimental to activity as was the case in previous series; in fact, in one case the activity was actually enhanced by such a change (#1091 and #822, table VI).

7. The styryl-quinoline compounds listed in table X have been studied ex-

TABLE IV

The relative antifilarial activity of a number of cyanines in which the quaternary nitrogen is present in a quinoline ring, and the tertiary nitrogen in a heterocyclic ring other than quinoline or pyrrole

CHEMOTHERAPY CENTER NO.	STRUCTURE	MINIMUM CURATIVE DOSE	MAXIMUM TOLERATED DOSE	THERAPEUTIC INDEX	IN VITRO INDEX
		mgm./kgm.	mgm./kgm.		
863		0.4	4.0	10.0	2.0
862		3.2	<6.4	<2.0	0.4
831		>8.0	8.0	<1.0	0.25
835		0.4	4.0	10.0	2.0

tensively in regard to chemotherapeutic activity in infested cotton rats by Bieter, Wright and their associates (8, 9). As in the case of the compounds previously discussed, these contain a quaternary and a tertiary nitrogen con-

TABLE V

The relative antifilarial activity of several (3-pyrrole)(2-pyridine) and (3-pyrrole)(4-pyridine) dimethincyanines against *L. carinii* of the cotton rat

CHEMOTHERAPY CENTER NO.	STRUCTURE	MINIMUM CURATIVE DOSE mgm./kgm.	MAXIMUM TOLERATED DOSE mgm./kgm.	THERAPEUTIC INDEX	IN VITRO INDEX
957		4.0	8.0	2.0	0.5
958		>1.6	<2.0	<1.0	0.5
825		>8.0	<16.0	<1.0	0.025
826		>4.0	8.0	<2.0	0.06

ected by a carbon chain whose bonds are alternately single and double. They differ, however, in that the tertiary nitrogen is not a member of the heterocyclic ring, but rather is a para-amino nitrogen. Compound #350, in which two ethyl

TABLE VI

The relative antifilarial activity of a number of cyanines in which the quaternary nitrogen is present in a benzimidazole ring

CHEMO- THER- APY CENTER NO.	STRUCTURE	MINI- MUM CURA- TIVE DOSE	MAXI- MUM TOLER- ATED DOSE	THERA- PEUTIC INDEX	IN VITRO INDEX
1091		mgm./ kgm. 3.2	mgm / kgm. <6.4	<2.0	0.4
822		>4.0	12.0	<3.0	1.25
838		0.8	<4.0	<5.0	0.25

groups were attached to the tertiary-N and an ethyl group was the substituent at the quaternary quinoline-N, was outstandingly active *in vitro*, though this high degree of activity was not retained in the cotton rat. The remaining

TABLE V

The relative antifilarial activity of several (3-pyrrole)(2-pyridine) and (3-pyrrole)(4-pyridine) dimethinacyanines against *L. carinii* of the cotton rat

CHEMICAL SERIAL NO.	STRUCTURE	MINI- MUM CURA- TIVE DOSE	MAXI- MUM TOLER- ATED DOSE	THERA- PEUTIC INDEX	IN VITRO INDEX
957		4.0	8.0	2.0	0.5
958		>1.6	<2.0	<1.0	0.5
825		>8.0	<16.0	<1.0	0.025
826		>1.0	8.0	<2.0	0.06

neeted by a carbon chain whose bonds are alternately single and double. They differ, however, in that the tertiary nitrogen is not a member of the heterocyclic ring, but rather is a para-amino nitrogen. Compound #350, in which two ethyl

TABLE VI

The relative antifilarial activity of a number of cyanines in which the quaternary nitrogen is present in a benzimidazole ring

CHEMO- THER- APY CENTER NO.	STRUCTURE	MINI- MUM CURA- TIVE DOSE	MAXI- MUM TOLER- ATED DOSE	THERA- PEUTIC INDEX	IN VITRO INDEX
		mgm / kgm.	mgm./ kgm.		
1091		3.2	<6.4	<2.0	0.4
822		>4.0	12.0	<3.0	1.25
838		0.8	<4.0	<5.0	0.25

groups were attached to the tertiary-N and an ethyl group was the substituent at the quaternary quinoline-N, was outstandingly active *in vitro*, though this high degree of activity was not retained in the cotton rat. The remaining

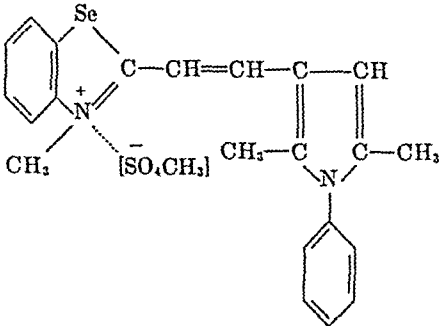
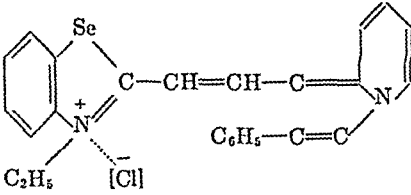
The relative antifilarial activity of a number of cyanines in which the quaternary nitrogen is present in a benzoxazole ring

TABLE VIII

The relative antifilarial activity of a number of cyanine dyes in which the quaternary nitrogen is present in a benzothiazole ring or a benzoselenazole ring

CHEMO-THER-APY CENTER NO.	STRUCTURE	MINI-MUM CURA-TIVE DOSE	MAXI-MUM TOLER-ATED DOSE	THERA-PEUTIC INDEX	IN VITRO INDEX
956		—	—	—	0.8
996		>4.0	8.0	<2.0	0.3
827		>2.0	<2.0	<1.0	1.0
839		0.8	<4.0	<5.0	0.5
849		—	—	—	0.3

TABLE VIII—Concluded

CHEMOTHERAPY CENTER NO.	STRUCTURE	MINIMUM CURATIVE DOSE	MAXIMUM TOLERATED DOSE	THERAPEUTIC INDEX	IN VITRO INDEX
828		— mgm./ kgm.	— mgm./ kgm.	—	0.05
841		>0.4	<0.8	<2.0	0.25

considerably less potency. Introduction of only one long alkyl radical was not as deleterious as introduction of two; for example, #764, with a methyl and an n-heptyl group on the tertiary nitrogen, retained an "in vitro index" of 0.5.

Number K-188 contained no tertiary nitrogen, and hence there was no resonating system between two nitrogens. This compound had an "in vitro index" of 0.02 which was low relative to the cyanines, but it was remarkable nonetheless, since it was the only compound studied which had an index of any significance despite the lack of the structural requirements described in the following paragraphs.

As has already been pointed out in previous communications (1, 2, 3), a high degree of antifilarial activity was observed with compounds possessing the resonating amidinium ion system, in which a quaternary nitrogen was separated from a tertiary nitrogen by a chain of atoms referred to as a "conjugated" chain, i.e., a chain whose members were joined by alternating single and double bonds. Tables I to X illustrate that marked antifilarial activity *in vitro* was not restricted to any particular ring or rings, and was observed regardless of whether both nitrogens (tables I to IX) or only one nitrogen (table X) was a part of a heterocyclic ring. If neither of the two nitrogens was part of a heterocyclic ring (table IX: #K-218), activity could be present, although at a much lower order of magnitude. On the other hand, if all the atoms linking the two nitrogens were part of a chain, and not a portion of a heterocyclic ring, activity

in vitro was completely abolished (table IX: #K-217). In the case of one compound, only one of the three linking atoms was part of the heterocyclic ring, yet antifilarial activity was retained (table IV: #831). In almost every case the atoms interposed between the two nitrogens were carbon; that this was not a requirement for activity is indicated by the fact that nitrogen could serve in place of carbon, as in #849 of table VIII which had an "*in vitro* index" of 0.3.

Compounds closely related to cyanines, but lacking an amidinium ion resonating system exhibited either a very low degree of antifilarial activity *in vitro* or none at all (#797 and #821, table I; #854 and #855, table III; #945 and #946, table IV; #912, #925, #1001, and #921, table XI). The last mentioned group of compounds was interesting also from the standpoint of being possible degradation products of the cyanines *in vivo*. It has been pointed out already that in the case of one compound (K-188, table X), some activity was observed in spite of the lack of a resonating amidinium ion system. This compound contained a quaternary nitrogen, but lacked the tertiary nitrogen to complete the structural requirements outlined.

A large number of well-known organic dyes resemble the cyanines chemically in their possession of a tertiary and a quaternary nitrogen separated by a "conjugated" chain of carbon atoms. Since the antifilarial action of the cyanines appeared to depend on these particular structural features, it seemed of interest to include some non-cyanine dyes in the study for purposes of comparison. None of these was found to be active *in vivo*, but a considerable number possessed a low degree of activity *in vitro*. For the sake of brevity these non-cyanine dyes are listed here without their structural formulae; in each case the "*in vitro* index" is given within parentheses: (a) triphenylmethane dyes: crystalviolet (0.025), brilliant green (0.1), malachite green (0.1), fuchsin (rosaniline) (0.1); (b) phenazothionium dyes: methylene blue (0), azur B (0); (c) phenazinium dyes: janus green (0.1), diethylsafranin (0.05), safranin-T (0.025); (d) other dyes: acridine orange (0.01), meldolas blue (0.003), shodamine B (0.0025), methylquinoline yellow (>0.002). Fluorescein, which possesses a "conjugated" chain between two oxygen atoms instead of two nitrogen atoms, exhibited no antifilarial activity *in vitro*.

Because some organic antimony compounds have been shown to exhibit antifilarial activity *in vivo* (4, 5, 7, 11), 'Fuadin' and potassium antimonyl tartrate were tested *in vitro*. They were only $\frac{1}{16}$ to $\frac{1}{128}$ as active as the reference cyanine (#348) in regard to their ability to inhibit the oxygen uptake of *L. carinii*.

In contrast to their inhibitory effect on the respiration of malaria parasites (9), quinine and atabrine were found not to interfere with the respiratory metabolism of *L. carinii*. This is in agreement with the observation that these two antimalarials have no chemotherapeutic action *in vivo* against *L. carinii* in the cotton rat.

F. SELECTION OF COMPOUNDS FOR FURTHER STUDY. It has already been pointed out that the inherent antifilarial activity of these compounds would operate in the infested animal host in proportion to the influence exerted by

TABLE IX

The relative antifilarial activity of a number of miscellaneous cyanine dyes against *L. carinii* of the cotton rat

CHEMO-THERAPY CENTER NO.	STRUCTURE	MINI-MUM CURA-TIVE DOSE	MAXI-MUM TOLER-ATED DOSE	THERA-PEUTIC INDEX	IN VITRO INDEX
830		mgm./kgm. >16.0	mgm./kgm. <16.0	<1.0	0.2
1021		>16.0	16.0	<1.0	0.3
1025		1.6	6.0	4.0	0.7
886		>8.0	8.0	<1.0	0.25
945		>20.0	>40.0	—	0.003

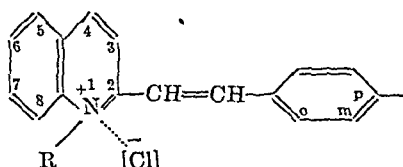
TABLE IX—Concluded

CHEMO-THERAPY CENTER NO.	STRUCTURE	MINI-MUM CURA-TIVE DOSE	MAXI-MUM TOLER-ATED DOSE	THERA-PEUTIC INDEX	IN VITRO INDEX
946		mgm./kgm. >16.0	mgm./kgm. >64.0	—	0
1113		>12.0	12.0	<1.0	0.2
1114		>16.0	16.0	<1.0	0.01
887		—	—	—	0.125
K217 (PD423)		—	—	—	0
K218		—	—	—	0

various host factors such as rate and extent of excretion, metabolic alteration, favorable and unfavorable distribution to the sites of occurrence of the invading parasite, etc. Since human filariasis involves a different host from that used

TABLE X

The relative antifilarial activity *in vitro* of a number of styryl-quinoline dyes against *L. carinii* of the cotton rat



CHEMOTHERAPY CENTER NO.	SUBSTITUENT GROUPS			IN VITRO INDEX
	R	p	6	
350	C ₂ H ₅	N(C ₂ H ₅) ₂	CH ₃	2.0*
759	CH ₃	N(C ₃ H ₇) ₂ (n)	CH ₃	0.4
763	CH ₃	N(C ₄ H ₇) ₂ (iso)	CH ₃	1.0
760	CH ₃	N(C ₄ H ₉) ₂	CH ₃	0.3
762	CH ₃	N(C ₈ H ₁₁) ₂	CH ₃	0.1
764	CH ₃	CH ₃	CH ₃	0.5
757	CH ₃		CH ₃	0.5
773	CH ₃			1.0
K-188 (PD-245)	CH ₃		—	0.02

* *In Vivo*: Min. Cur. Dose = 1.6 mgm./kgm.

Max. Tol. Dose = <8.0 mgm./kgm.

Therapeutic Index = <5.0.

in this study, and a different site of occurrence of the invading parasite, namely the lymphatic system, it is obvious that the only infallible method of selecting the best compound would involve the testing against the human disease of at least those compounds shown to have a high degree of antifilarial action *in vitro*.

The impracticability of such a procedure is obvious. To minimize the modifying factors which might be imposed by a different host, and a different site of occurrence of the invading parasite, the compound selected from this study for further investigation should possess a high degree of activity against *L. carinii*, both *in vitro* and *in vivo*. Compound #863 (table IV) appeared to possess the best combination of indices, since it was twice as active as the reference compound (#348) *in vitro* and, in addition, had a high (absolute) therapeutic index. However, since many other qualifications would be demanded of a compound

TABLE XI

The relative antifilarial activity *in vitro* of a number of possible degradation products of cyanine dyes against *L. carinii* of the cotton rat

CHEMOTHERAPY CENTER NO.	STRUCTURE	IN VITRO INDEX
912		0
925		0
1001		0.001
921		0.003

to be administered to human beings, it seemed unwise to eliminate all but one compound at this early stage of the investigation. Furthermore, 12 compounds had therapeutic indices of 10 to 15. Only 4 of these showed less than 50 per cent of the activity of #348 *in vitro*, while 5 were more active than the reference compound. These compounds were numbers 712, 713, 798, 824, 835, 871, 863, 943, 963, 964, 965 and 967. All these were retained temporarily for further study in regard to possible therapeutic utility in human filariasis, with the exception of #712. This compound was omitted because of its very low solubility which accounts at least partly for its low degree of antifilarial activity

in vitro. This property also rendered an accurate measure of its chemotherapeutic activity impossible, since suspensions, rather than solutions, were injected, and residual drug was invariably found in the peritoneal cavity at autopsy following the administration of large doses.

An additional factor, unanswered by any of the above considerations, but of paramount importance in transferring a drug of this group from the laboratory to the clinic, was its relative antifilarial potency for *L. carinii* of the cotton rat and for *W. bancrofti* of man. This would depend largely on the degree of similarity or dissimilarity in the metabolic characteristics of these two parasites. The impossibility of obtaining *W. bancrofti* for studies *in vitro* made it necessary to postpone the appraisal of this critical factor until the time of clinical trial of the particular cyanine dye eventually selected for this purpose.

SUMMARY AND CONCLUSIONS

(1) In a routine "screening" program, the cyanine dye, (1-amy-2,5-dimethyl-3-pyrrole)(1,6-dimethyl-2-quinoline) dimethincyanine chloride (Chemotherapy Center #348) was found to possess marked chemotherapeutic properties against the filarial parasite, *Litomosoides carinii*, of the cotton rat.

(2) In doses which are only a fraction of those maximally tolerated this compound was able to produce complete cures when administered intraperitoneally every eight hours for 6 days, once daily for 5 days, once daily for 3 days. Although a cure could be obtained with a single dose, the size of the latter approached that of the acutely lethal dose.

(3) This compound inhibited the oxidative metabolism of adult *L. carinii*, in dilutions as high as 1:40 million.

(4) A large number of related cyanine dyes were studied with regard to both their curative activity and their antimetabolic effect. The particular chemical structure which appears to be essential for the antifilarial effect, and the effect of various structural modifications on antifilarial activity, are discussed.

(5) On the basis of a high degree of antifilarial activity both *in vivo* and *in vitro*, eleven compounds were selected for further study with the object of an eventual selection of one for clinical trial in human filariasis.

REFERENCES

1. WELCH, A. D., PETERS, L., BUEDING, E., VALK, A. D., JR., AND HIGASHI, A.: *Science*, 105: 486, 1947.
2. (a) WELCH, A. D., PETERS, L., BUEDING, E., VALK, A. D., JR., AND HIGASHI, A.: *Fed. Proc.*, 6: 382, 1947.
(b) PETERS, L., HIGASHI, A., AND WELCH, A. D.: *Fed. Proc.*, 6: 362, 1947.
(c) VALK, A. D., JR., PETERS, L., AND WELCH, A. D.: *Fed. Proc.*, 6: 379, 1947.
(d) BUEDING, E.: *Fed. Proc.*, 6: 312, 1947.
3. (a) BUEDING, E.: *Annals N. Y. Acad. Sciences*, 50: 115, 1948.
(b) PETERS, L.: *Annals N. Y. Acad. Sciences*, 50: 117, 1948.
4. CULBERTSON, J. T., AND ROSE, H. M.: *THIS JOURNAL*, 81: 189, 1944.
5. CULBERTSON, J. T., AND PRICE, E.: *THIS JOURNAL*, 87: 181, 1946.
6. CULBERTSON, J. T., ROSE, H. M., AND OLIVER-GONZALEZ, J.: *Am. J. Trop. Med.*, 25: 271 and 403, 1945.

7. CULBERTSON, J. T.: *Annals N. Y. Acad. Sciences*, 50: 73, 1948.
8. (a) BIETER, R. N., CUCKLER, A. C., LITCHFIELD, J. T., JR., BREY, T. E., AND WRIGHT, H. N.: *Fed. Proc.*, 6: 310, 1947.
(b) WRIGHT, H. N., CUCKLER, A. C., CRANSTON, E. M., AND BIETER, R. N.: *Fed. Proc.*, 6: 388, 1947.
(c) CRANSTON, E. M., CUCKLER, A. C., LITCHFIELD, J. T., JR., BREY, T. E., WRIGHT, H. N., AND BIETER, R. N.: *Fed. Proc.*, 6: 318, 1947.
9. WRIGHT, H. N., LITCHFIELD, J. T., JR., BREY, T. E., CRANSTON, E. M., CUCKLER, E. M., CUCKLER, A. C., AND BIETER, R. N.: *Annals N. Y. Acad. Sciences*, 50: 115, 1948.
10. OTTO, G. F., AND MAREN, T. H.: *Annals N. Y. Acad. Sciences*, 50: 39, 1948.
11. BROWN, W. H.: *Annals N. Y. Acad. Sciences*, 50: 51, 1948.
12. BROOKER, L. G. S.: *Advances in Nuclear and Theoretical Organic Chemistry*. R. E. Burk and Oliver Grummitt. Interscience Publishers, Inc., New York, N. Y. 1945, Chapter IV, pp. 63-136.
13. BROOKER, L. G. S.: *Annals N. Y. Acad. Sciences*, 50: 108, 1948.
14. SILVERMAN, M., CEITHMAL, J., TALIAFERRO, L. G., AND EVANS, E. A., JR.: *Jour. Inf. Dis.*, 75: 212, 1944.
15. BUEDING, E.: *Jour. Exp. Med.*, 89: 107, 1949.
16. PETERS, L., BUEDING, E., VALK, A. D., JR., HIGASHI, A., AND WELCH, A. D.: To be published.

A PHARMACOLOGIC COMPARISON OF HEXAETHYL TETRAPHOSPHATE (HETP) AND TETRAETHYL PYROPHOSPHATE (TEPP) WITH PHYSOSTIGMINE, NEOSTIGMINE AND DFP¹,²

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The parasympathomimetic properties of a number of alkyl polyphosphate compounds have been established in the course of several investigations into the pharmacologic actions of these agents. DuBois and Mangun (1) were the first to report the cholinesterase inhibiting effect of hexaethyl tetraphosphate (HETP). They found that HETP was about four times as potent as diisopropyl fluorophosphate (DFP) in inhibiting rat brain cholinesterase activity *in vitro*. Hagan and Woodard (2) studied the toxicity of HETP for several species. Deichmann and Witherup (3) reported that tetraethyl pyrophosphate (TEPP) was about two and a half times as toxic as HETP when given orally to rats. Mangun and DuBois (4) included TEPP in a comparison of the potency of these compounds with that of DFP in inhibiting rat brain cholinesterase *in vitro*. They found that a 50 per cent inhibition was effected by $4 \times 10^{-9} M$ TEPP, by $1.6 \times 10^{-8} M$ HETP, and by $6.3 \times 10^{-8} M$ DFP under the same conditions. Roeder and Kennedy (5) compared the effects of stimulating the afferent nerves entering the sixth abdominal ganglion of the cockroach in the presence of $6 \times 10^{-5} M$ DFP and of $2.5 \times 10^{-7} M$ HETP. They observed similar changes consisting of prolonged after discharge in the giant fibers of the ventral nerve cord and ganglionic transmission alternating from facilitation to temporary block. Koppanyi, Karczmar and King (6) gave evidence that TEPP increases the sensitivity of sympathetic ganglia at certain dosage levels. DFP and physostigmine were shown to have similar actions. Burgen *et al.* (7) compared the effects of TEPP and HETP on isolated tissues and on the chloralosed cat. Dayrit, Manry, and Seevers (8) made some detailed observations on the pharmacologic action of HETP with special reference to its cardiovascular, respiratory, and anticholinesterase effects in dogs.

The purpose of this study was to compare directly, under the same experimental conditions, some of the pharmacodynamic actions of HETP and TEPP with those of three other better known cholinesterase inhibitors, e.g., physostigmine, neostigmine, and DFP, in order to add to the information which might form the basis for selecting from these agents one which would serve best in

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specific cases as a research tool or as a therapeutic agent. The comparative actions of these five anticholinesterase agents were observed on the blood pressure, electrocardiogram, isolated heart, and small intestine of various species of experimental animals.

EXPERIMENTAL. *Effects on Blood Pressure.* Forty-six cats weighing 2 to 4 kgm. and 25 dogs weighing 7 to 12 kgm. were used in this study. The cats were anesthetized with 180 mgm./kgm. and the dogs with 150 mg./kgm. of Na-phenobarbital intraperitoneally. The trachea was cannulated in all animals and a Palmer positive pressure artificial respiration pump was used when required. Blood pressure changes were recorded by a mercury manometer connected to the right carotid artery. The right femoral vein was used for injection. Chest movements were recorded by means of a tambour actuated by a pneumograph placed on the chest.

A single intravenous injection of 0.3 mgm./kgm. HETP, 0.1 per cent in saline, generally produced a 30 to 40 mm. rise in blood pressure which reached its maximum in 1 to 2 minutes and subsided to normal in 7 to 10 minutes. This effect was not accompanied by any significant change in heart rate, or in respiratory rate or amplitude. A second injection of the same dose, given 15 minutes to 4 hours after the first, produced a depressor response of about the same magnitude and duration (fig. 1). A third such dose also elicited a fall in blood pressure, this time attended by a marked slowing of the heart and a fatal respiratory paralysis (fig. 1). Atropinized cats (1 mgm./kgm.) gave successive pressor responses to these doses of HETP and survived three to five times the amount tolerated by unatropinized animals. Larger doses of atropine (10 mgm./kgm.) afforded greater protection against HETP toxicity and enhanced the rise in blood pressure. The responses to 0.1 mgm./kgm. of TEPP or neostigmine and 0.2 mgm./kgm. of physostigmine were equivalent to those to 0.3 mgm./kgm. of HETP under these conditions. Furthermore, these drugs could be used interchangeably in a series of injections eliciting the above described sequence of blood pressure changes, i.e. using the above doses the pressor response to any one was followed by a depressor response to the subsequent injection of another. The blood pressure changes produced by DFP alone were slight and variable, but 0.6 mgm./kgm. of DFP would reverse the pressor response to the subsequent injection of any of the above agents.

When smaller doses of HETP (0.15 mgm./kgm.) were injected intravenously, successive rises of 10 to 15 mm. blood pressure could be elicited until the fourth or fifth injection which resulted in a depressor response. This sequence also resulted from comparable doses of TEPP, neostigmine, or physostigmine and here again these drugs could be used interchangeably in the series of injections.

The injection of 0.6 mgm./kgm. or more of HETP produced first a 30 to 40 mm. rise in blood pressure lasting 2 to 3 minutes followed by a precipitous fall accompanied by a marked slowing of the heart and a great increase in pulse pressure suggesting strong vagal action with A-V block (fig. 2). This picture persisted for as long as 30 minutes and artificial respiration was frequently required to prevent death. This change was not influenced by sectioning both vagi but

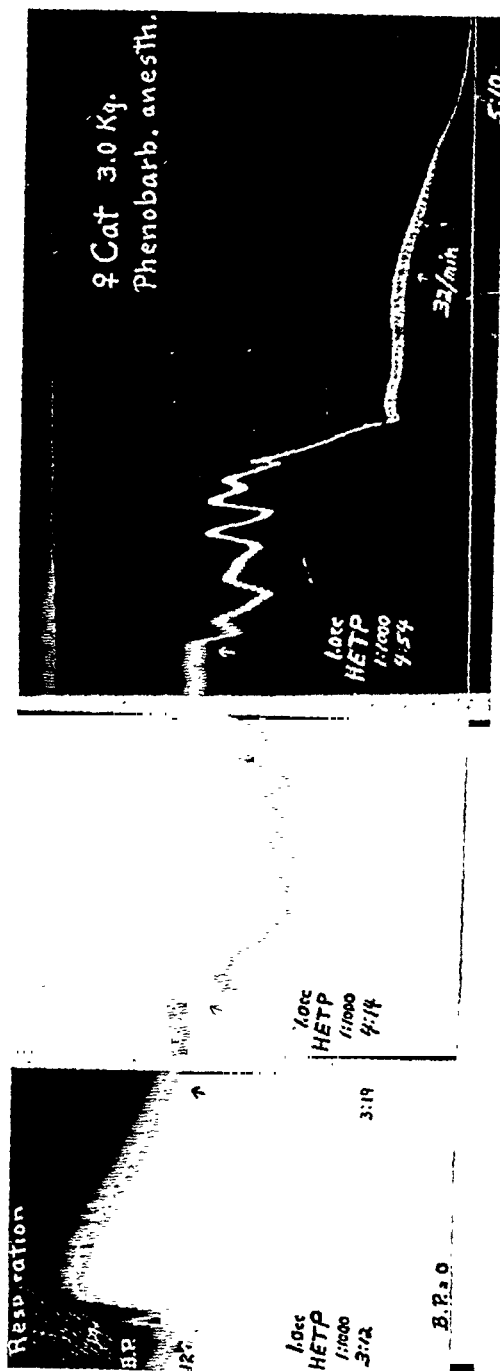


FIG. 1. BLOOD PRESSURE RESPONSES TO SUCCESSIVE DOSES OF 0.33 MG./KG. OF HETP SHOWING PRESSOR RESPONSE FROM FIRST INJECTION AND DEPRESSOR RESPONSES FROM SECOND AND THIRD INJECTIONS
Note time intervals and terminal respiratory paralysis

was abolished by 1.0 mgm./kgm. of atropine. The approximate threshold doses required to elicit this response were 0.2 mgm./kgm. of TEPP or neostigmine, 0.4 mgm./kgm. of physostigmine and 1.0 mgm./kgm. of DFP. In the case of DFP this action was without the preliminary rise in blood pressure and did not occur until about ten minutes after injection. Two successive doses of 0.3 mgm./kgm. of HETP potentiated the depressor action of acetylcholine about ten-fold. Similar potentiation was produced by comparable doses of each of the other four drugs tested.

The pressor effects of physostigmine and neostigmine have never been satisfactorily explained (9, 10, 11). Experiments were therefore conducted to attempt

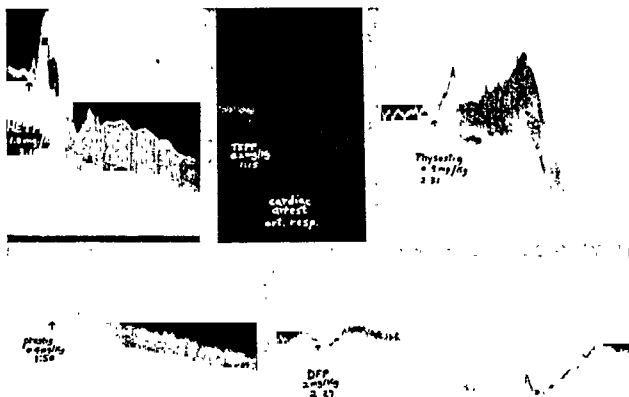


FIG. 2. BLOOD PRESSURE RESPONSES IN ANESTHETIZED DOGS (SODIUM PHENOBARBITAL) TO SINGLE LARGE DOSES OF EACH OF FIVE CHOLINESTERASE INHIBITORS STUDIED

Showing marked primary pressor responses (except with DFP), and precipitous fall in blood pressure and great increase in pulse pressure (strong vagal action). Note delayed action of DFP and restorative effect of 20 mgm./kgm. of procaine.

to elucidate the mechanisms involved in these blood pressure changes. Complete nicotization of the animal by giving a series of injections of nicotine sulfate at short intervals (1 minute or less) until the pressor response to this drug was completely abolished did not influence the pressor responses to HETP, TEPP, neostigmine or physostigmine. This indicated that any ganglionic effect was not nicotine-like. These drugs also elicited their typical pressor responses in adrenalectomized animals. Bilateral nephrectomy, vagotomy, evisceration or decapitation also had no definite effect on the pressor responses.

Small doses of the sympatholytic agent dibenamine HCl (5.0 mgm./kgm.), which reverse the pressor effect of injected epinephrine, did not influence the

pressor response to HETP or nicotine. However, large doses of dibenamine HCl (30 mgm./kgm.) reversed the pressor response to both these drugs. This difference in the effects of adrenolytic and sympatholytic doses of dibenamine further supports the conclusion that liberated epinephrine does not contribute markedly to the pressor responses to these drugs.

It is known (12) that the injection of procaine produces significant changes in the excitability of the autonomic nervous system. The depressor response to injected acetylcholine is greatly reduced, the pressor response to nicotine is reduced and the pressor response to epinephrine is increased. In a dose of 20 mgm./kgm. procaine was seen to exert an atropine-like effect in preventing the depressor effects of HETP and TEPP and in abolishing the bradycardia and restoring a normal heart action after the larger doses of these drugs. Procaine was much less effective than atropine in this respect and the duration of its restorative and the extent of its protective actions were much less than that of atropine.

Haimovici and Pick (13) reported that thiamine blocks the pressor action of nicotine. We found that 200 mgm./kgm. of thiamine-HCl given slowly in divided doses would effectively block nicotine and would markedly suppress the pressor responses to HETP and TEPP. Thiamine also blocks the stimulating effect of nicotine on striated muscle as shown on the frog rectus abdominis by Unna and Pick (14). They concluded that this action of thiamine was on the myoneural junction.

Electrocardiographic Changes. The Sanborn string galvanometer electrocardiograph was employed on 22 dogs. In order to obtain suitable records consistently the dogs were anesthetized with 150 mgm./kgm. of Na-phenobarbital. Observations were made using the conventional Leads I, II, and III.

Electrocardiograms taken following single intravenous doses (fig. 3) of the drugs being compared revealed that similar profound changes in cardiac rhythm were produced by all of the agents. Electrocardiographic findings following HETP have been described by Dayrit, Manry, and SeEVERS (8) who observed a marked sinus bradycardia without an increase in the P-R interval, a partial to complete A-V block, and with larger doses a disappearance of the P waves. In addition to these changes we regularly observed, with the five drugs studied, marked changes in the T wave, such as inversion in all leads, or exaggeration with or without inversion especially in leads II and III. On one occasion inversion was seen in alternating cardiac cycles in all three leads after TEPP. Small doses were capable of producing a pressor effect with no visible electrocardiographic changes. Moderate doses produced a slowing which sometimes developed into varying degrees of A-V block. Large doses frequently precipitated a sudden A-V block without preliminary slowing, and in this case the P wave often disappeared and there was an increase in the magnitude and variability of the T wave.

The A-V block was manifested in almost every possible way. A-V ratios of 2:1 and 3:1 were frequently seen. More commonly however such blocks soon developed into complete A-V dissociation with approximate ratios ranging from

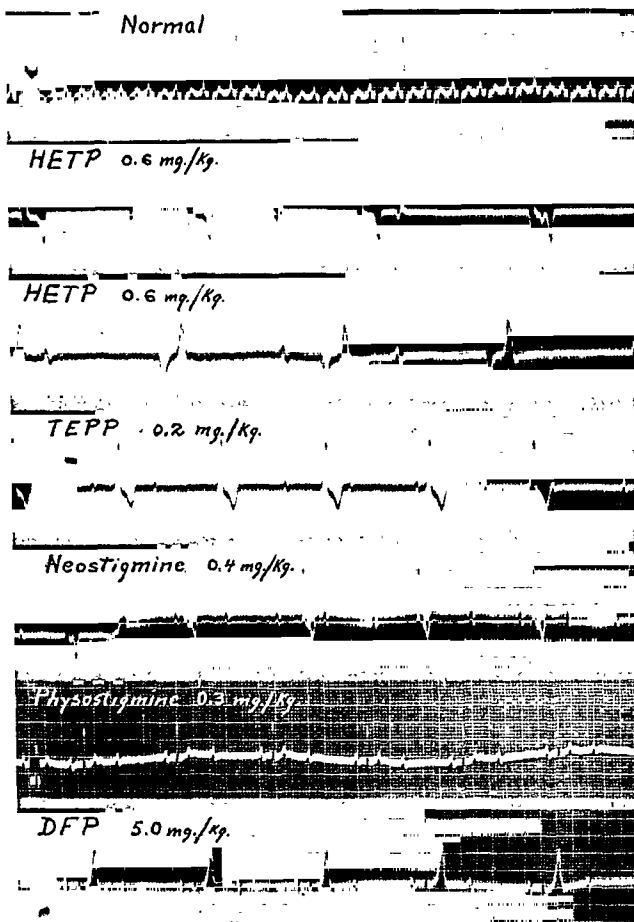


FIG. 3. ELECTROCARDIOGRAMS OF DOGS ANESTHETIZED WITH SODIUM PHENOBARBITAL.
Leads indicated by white blocks at upper edge of each record

2:1 to 5:1. There was no evidence that there was any difference in the cardiac effects of HETP, TEPP, physostigmine, neostigmine, and DFP, except for the

delayed onset of the action of the DFP. Since these changes were not affected by vagotomy but were prevented or abolished by atropine or procaine and prevented by thiamine given previously, it is suggested that the site of action is at or peripheral to the intrinsic synapses of the parasympathetic innervation of the heart. Since the isolated heart was found to be highly resistant to all these anticholinesterase agents (see below) it appears likely that the observed effects on the heart of the intact animal are due to an accumulation of acetylcholine coming to the heart by way of the circulation.

Effects on the Isolated Rabbit Heart. Experiments were conducted upon 40 isolated rabbit hearts perfused with oxygenated Ringer-Locke solution at a pressure of 50 to 70 cm. of perfusion solution at 38°C. The aorta was cannulated and the perfusion fluid forced by gravity into the coronary arteries. Observations were made on the effects of the anticholinesterase agents administered (a) as single injections into the perfusion line immediately adjacent to the heart and (b) in various concentrations in the stock perfusion fluid.

The results of these tests indicated that the isolated heart exhibits little specific sensitivity to HETP or TEPP, as has been reported for physostigmine (15), DFP (16), and neostigmine (9). The injection of 1.0 mgm. (1.0 cc. of 1:1,000) of HETP directly into the cannula leading to the aorta and coronary circulation produced no apparent change in heart activity. In the intact cat or dog profound cardiac changes were always observed upon intravenous injection of 0.6 mgm./kgm. Following the injection of 5 mgm. (0.25 cc. of 1:50) of HETP, the amplitude and coronary flow of the isolated heart were reduced to about one-half normal for about three minutes without any marked change in rate. A good heart action was restored after this time but the amplitude did not return to normal (fig. 4). A second injection of the same dose produced the same reduction in amplitude but this time an increase in rate of coronary flow occurred. However, the heart did not recover from the second dose and heart action stopped in about 15 minutes. These changes in amplitude and coronary flow were not influenced in any way by the presence of atropine in the Ringer-Locke solution in a concentration of 2 mgm./liter (fig. 4). The responses of the isolated rabbit heart to TEPP, physostigmine, neostigmine, and DFP administered in this way were qualitatively the same as those described for HETP.

The maximum concentrations of HETP, TEPP, physostigmine, and DFP tolerated for one hour without effect on the isolated heart were determined when these agents were present in the oxygenated Ringer-Locke perfusion solution. These concentrations are shown in table 1 along with an estimate of the extent to which they reduce the amount of acetylcholine necessary to produce a minimal and evanescent slowing of the heart. These results show no apparent correlation between the maximum tolerated perfused concentrations and the reported cholinesterase inhibiting capacity or the toxicity of the various agents. Furthermore there appeared to be no relation between the tolerated concentrations and the degree of the potentiation of the response to acetylcholine.

The duration of the cardiac depression caused by minimal effective doses (0.2 cc. 1:50,000) by acetylcholine was increased about five times by previous treat-

ment with the anticholinesterase agents. The potentiation of the action of acetylcholine was less following temporary perfusion or single injections of sub-toxic doses of HETP than during continuous perfusion with the drug. However, the isolated heart from a rabbit sacrificed one day after receiving a

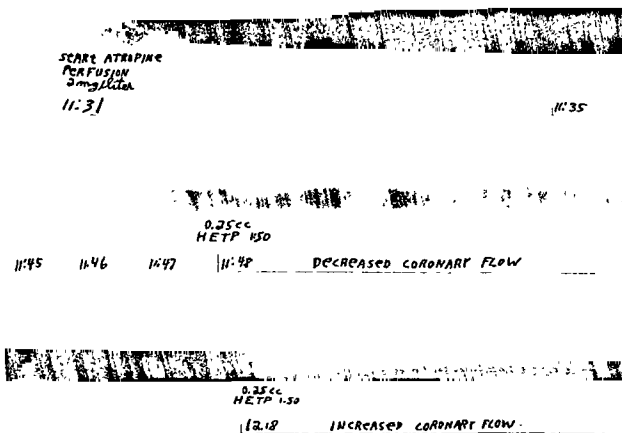


FIG. 4. ISOLATED RABBIT HEART PERFUSED WITH LOCKE-RINGER SOLUTION
Atropine did not influence the changes produced by HETP

TABLE 1

Maximum perfusion concentrations of anticholinesterase agents tolerated for one hour by isolated rabbit heart and resultant potentiation of the sensitivity to acetylcholine

ANTI-CHOLINESTERASE AGENT	MAX. TOLERATED CONC $\times 10^{-4} M$	APPROXIMATE ACETYL- CHOLINE POTENTIATION
DFP	0.38	40 times
HETP	2.0	40 times
TEPP	3.5	40 times
Physostigmine	5.0	20 times

dose of 0.3 mgm./kgm. HETP showed about a 20-fold decrease in the dose of acetylcholine usually required to elicit a minimal response.

These studies indicated no qualitative differences in the actions of the five drugs used on the isolated rabbit heart. Relatively large amounts of each were required to produce what appeared to be a direct toxic effect. This action is considered totally independent of the anticholinesterase properties of the agents.

Effects on the Small Intestine of the Rabbit. The comparative actions of the

five agents under investigation were studied using two hundred and thirty washed strips of rabbit ileum suspended in oxygenated Ringer-Locke solution in 100 cc. glass baths kept at a constant temperature of 37.5°C. Parallel tests were usually carried out on two intestinal strips recording simultaneously. All tests were made using gut specimens removed from a freshly killed animal. In a few experiments the motility of the gut *in situ* was recorded by means of a device described by Jackson (17).

HETP, TEPP, neostigmine, physostigmine, and DFP produced qualitatively the same changes in the activity of the isolated rabbit ileum. The minimal concentration producing a characteristic gradual increase in tone and a delayed change from the normal pendular rhythm to a peristaltic type of rhythm was determined for each drug. A departure from the pendular activity occurred abruptly 15 to 20 minutes after the introduction of the drug into the bath and the new peristaltic rhythm was characterized by a series of profound relaxations and strong contractions alternating at regular intervals of 1 to 3 minutes (fig. 5). This peristaltic type of rhythm persisted as long as recordings were made, 3 to 4 hours in some cases. The reversibility of these changes of gut activity was tested by repeated washing. It was found that although these effects of physostigmine and neostigmine were easily reversed by washing, with a return to normal pendular activity, the effects of HETP, TEPP, and DFP could not be reversed by washing. These findings are summarized in table 2. The potency of these drugs on the rabbit gut was of the same order as their reported effectiveness as cholinesterase inhibitors (6). The effects of HETP on four intestinal strips from two guinea pigs were essentially the same as those observed on the rabbit ileum.

Atropine sulfate in a concentration of 3×10^{-8} M restored normal tone and activity following the onset of the changes induced by a minimal effective dose of each anticholinesterase drug (fig. 6A). The magnitude of the antagonizing molar concentration of atropine (10^{-8}) is of the same order as that of the minimal effective concentrations of all five agents. Larger doses of the cholinesterase inhibitors (100 to 1000 times the minimum effective dose) broke through the protection afforded by the above concentration of atropine but did not completely abolish the normal pendular rhythm. The fact that a given concentration of atropine blocked the action of a wide range of doses of a cholinesterase inhibitor probably indicates that the atropine is antagonizing endogenous acetylcholine which may be independent of the dose of the inhibitor over this range.

An increase above the minimal effective concentration of the anticholinesterase drug in the bath resulted in a more rapid onset of the typical changes in the rhythm of the intestinal strip. A one hundred-fold increase in concentration gave rise to an immediate effect. One-tenth the minimal effective dose of HETP potentiated the response of the gut to acetylcholine about 100 times. The administration of acetylcholine into the bath after a minimal effective dose of HETP hastened the onset of effect of the latter. Acetylcholine itself, however, did not induce the changes in rhythm described above for the cholinesterase inhibitors.

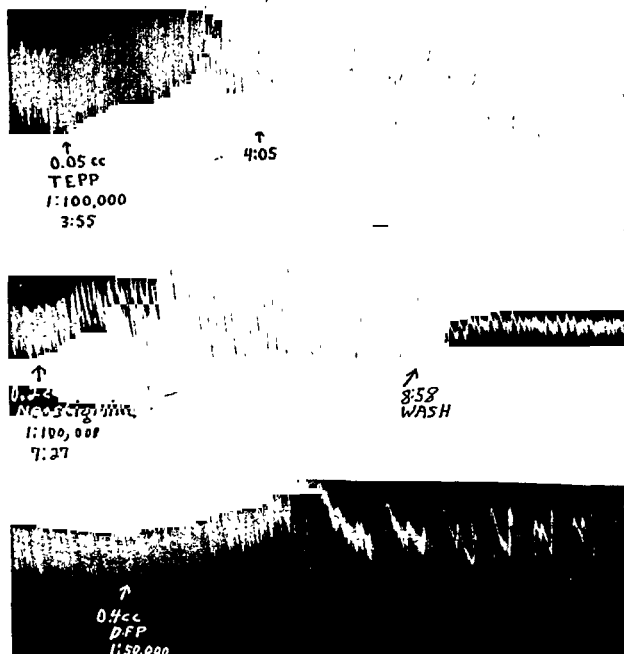


FIG. 5. ISOLATED RABBIT ILEUM SUSPENDED IN 100 CC. BATH OF OXYGENATED LOCKE RINGER SOLUTION TO WHICH THE ABOVE INDICATED DOSES WERE ADDED

Note slow rise in tone, delayed sudden change from pendular to peristaltic rhythm, and reversibility of the effect of neostigmine by washing.

TABLE 2

Minimal effective concentrations of anticholinesterase drugs producing changes in rhythm of isolated rabbit ileum, and the influence of washing

AGENT	MINIMAL EFFECTIVE CONCENTRATION $\times 10^{-3}$		IS EFFECT REVERSED BY WASHING?
	By Weight	On Molar Basis	
TEPP.	1.0	3.5	No
Physostigmine.	1.4	3.5	Yes
Neostigmine.	1.4	4.3	Yes
HETP.	5.0	9.8	No
DFP	10.0	54.3	No

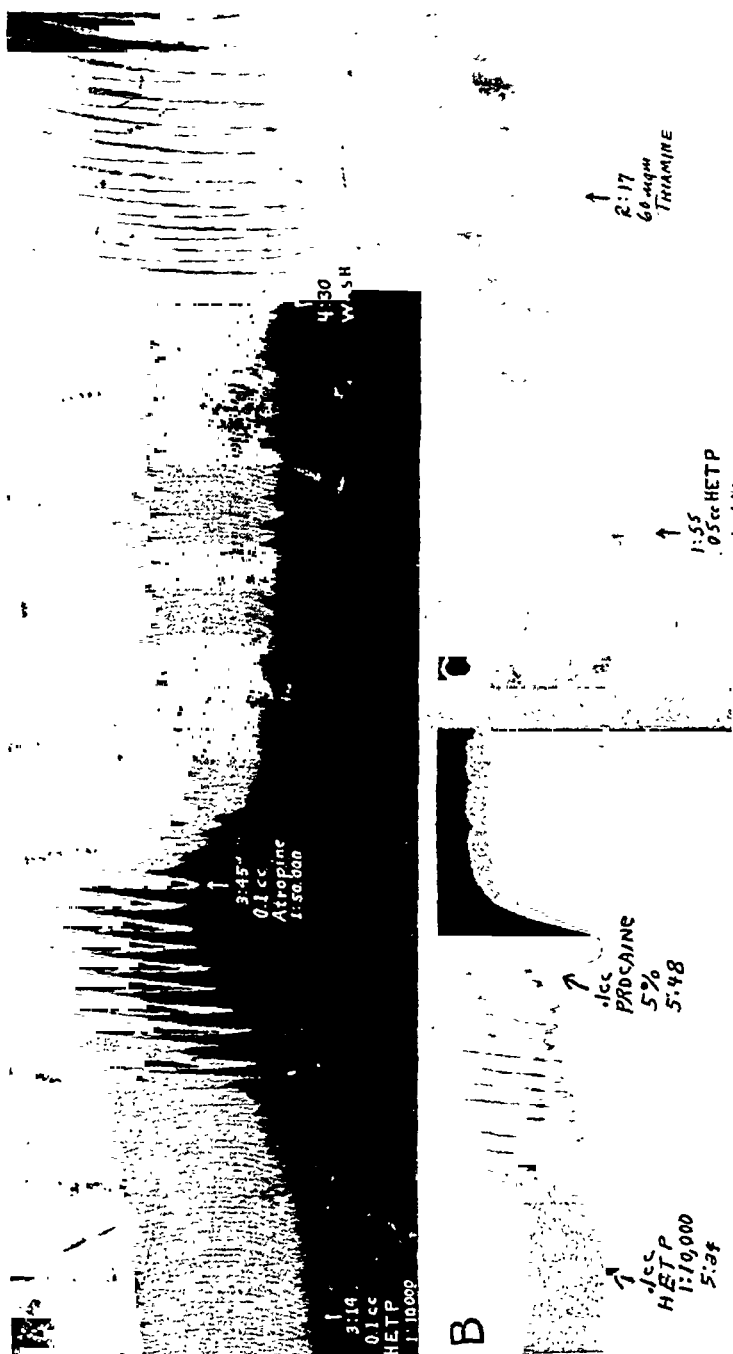


FIG. 6. ISOLATED RABBIT ILEUM AS IN FIG. 5

(a) The reversal to pendular rhythm caused by atropine was removed by washing. (b, c) Procaine and thiamine caused reversal to pendular rhythm but did not block rise in tone.

The nature of the response of the intestinal strip to these drugs was further studied in relation to nicotine, procaine, and thiamine. A series of injections of nicotine (0.2 cc. of 1:10,000 into a 100 cc. bath) at about five minute intervals resulted in successively smaller fleeting increases in tone until at the end of the fourth injection the intestinal strip was no longer responsive. Here it is supposed that the nicotine paralyzed nerve tissue only (ganglion cells), for the normal pendular activity, which is thought to be independent of nerve tissue, was not affected. When an effective dose of HETP was then given, there resulted an increase in tone and a decrease in amplitude but not distortion of rhythm. If a series of nicotine injections was introduced into the bath after the full HETP effect was produced, the pendular type of activity was restored.

Procaine-HCl in a concentration of 5×10^{-4} maintained normal pendular activity if given before or restored it if given after an effective dose of any of the cholinesterase inhibitors studied (fig. 6B). The action of procaine was like that of atropine except that the rise in tone was not blocked. Procaine merely preserved the pendular rhythm. Procaine in doses which had little effect on gut activity blocked the effects of nicotine and reduced the response to acetylcholine. The significance of this will be discussed later.

Thiamine-HCl in a concentration of 5×10^{-4} was also effective in reversing the effects of minimal effective concentrations of the cholinesterase inhibitors (fig. 6C). Unna and Pick (18) have shown that thiamine will block the action of nicotine on the gut. Following HETP, TEPP, and DFP the blocking actions of thiamine, atropine or procaine could be reversed by flushing out the bath; this reestablished the typical peristaltic rhythm previously induced by these agents. This effect in the case of atropine is demonstrated in fig. 6A.

Small doses of HETP (less than 0.15 mgm./kgm.) which did not elicit marked blood pressure responses produced a marked contraction of the small intestine *in situ* in the cat. The increase in tone and increased peristaltic action under these conditions lasted only about 20 minutes. Similar responses were observed with comparable doses of each of the other four cholinesterase inhibitors. Apparently the distribution of the drug in the intact animal is such that the concentration attained in the intestine has only a fleeting action.

Preliminary tests showed that the isolated uterus of the rabbit or guinea pig was not responsive to the drugs studied here.

DISCUSSION. The results of this comparison of five potent cholinesterase inhibitors discloses an extensive parallelism between these drugs with respect to certain pharmacologic actions. With only one exception, namely the failure of DFP to elicit a consistent pressor response upon intravenous injection in the dog and cat, all five agents exhibited the same pharmacologic properties when tested upon the circulation of anesthetized dogs and cats, upon the electrocardiogram in the anesthetized dog, and upon the isolated heart and intestinal strip of the rabbit. Since these five drugs gave qualitatively similar responses, it is not unlikely that a common site and mode of action is the causal factor in producing these responses in the physiologic systems tested. The manner in which the effects are influenced by other pharmacologic agents and the fact that

these drugs are interchangeable in eliciting the characteristic sequence of blood pressure responses with repeated doses in the intact animal constitutes further support for a common mode of action.

It is readily conceivable that in the case of the isolated intestine, this mode of action is based solely on the anticholinesterase activity of the drugs. Here it seems significant that the minimal effective concentration of these agents was of the same order as the reported 50 per cent cholinesterase inhibitory concentrations (6). In the case of the isolated heart, however, where there is no accumulation of acetylcholine, the observed effects of these cholinesterase inhibitors could hardly be attributed to an inactivation of cholinesterase. Their actions here were still markedly similar, however, though the amounts necessary were so large that the effects could not possibly be of significance in intact animal studies.

The pressor effects of these agents have been described (6, 9, 10, 11) but they have not been satisfactorily explained. Koppanyi *et al.* (6) offer evidence that the cholinesterase inhibitors alter the sensitivity of sympathetic ganglia. In confirmation of the results of other workers we were not able to diminish significantly the responses to these drugs by adrenalectomy or evisceration. Nicotization also had no influence. However, we succeeded in reversing the pressor effects of HETP, TEPP, physostigmine and neostigmine by large sympatholytic doses of dibenamine. Smaller adrenolytic doses of dibenamine did not modify the pressor responses. These facts constitute evidence that the drugs either are able to stimulate sympathetic ganglia which are presumably paralyzed by nicotine, or can exert a peripheral sympathin-like action which requires large doses of dibenamine to neutralize. Neither of these possible actions could be considered referable to an inhibition of cholinesterase.

A very rapidly developed apparent tachyphylaxis to the pressor action of these agents was observed. This was not a true tachyphylaxis, however, for the atropinized animal gave successive pressor responses to repeated moderate doses until a respiratory paralyzing dose accumulated in the body. It is logical to suppose that small pressor doses do not have sufficient anticholinesterase activity to result in any obvious parasympathetic stimulation, but with repeated doses the cumulative antiesterase action becomes dominant and any persisting peripheral vasoconstriction is masked.

Why DFP does not elicit a pressor response like the other drugs studied is not known. Some light on this question may be furnished by the fact that DFP has a greater affinity for the non-specific plasma cholinesterase than for the specific true cholinesterase of brain and peripheral nerve (21, 22). It is well known that very low plasma esterase levels are not necessarily reflected in pharmacodynamic responses. It is significant that DFP, which does not exhibit a pressor action, readily contributes to the induction of tachyphylaxis to the pressor action of HETP, TEPP, physostigmine and neostigmine.

In the intact animal the cardiac effects of the agents studied may be explained by the accumulation of acetylcholine. Since these effects are not influenced by vagotomy but are abolished or prevented by atropine, and do not occur in the

isolated heart, one must conclude that the accumulated acetylcholine in this case comes to the heart by way of the circulation, and is not the product of a stimulation of the parasympathetic ganglia in the heart muscle by the antiesterases themselves. It is possible, however, that the acetylcholine so accumulated may induce such a ganglionic stimulation, thus resulting in the liberation of more acetylcholine at the nerve endings. The comparatively weak blocking action of procaine, compared with that of atropine, against the cardiac effects of the esterase inhibitors, may be explained on this basis. It may be presumed that procaine eliminates the action of acetylcholine on the synapses but affects to a lesser extent (19) the action on the cardiac effector cell.

In the case of the isolated rabbit heart it was observed that the maximum concentrations of the antiesterase drugs tolerated for one hour without effect were of the order of 10,000 times more than the minimal concentrations which would produce an increase in tone and a change in rhythm of the isolated rabbit intestine. This difference would be expected from the fact that acetylcholine is continually being formed in the isolated gut (23) but not in the isolated heart. The atropine-like action of procaine and thiamine in reestablishing a normal pendular rhythm after its distortion by the esterase inhibitors suggests that ganglionic stimulation plays a role in the effects of the latter on the gut, since procaine and thiamine are thought to interfere with nerve transmission but not with the action of acetylcholine on the effector cell. It is supposed that such a ganglionic stimulation results from the accumulated acetylcholine and not directly from the esterase inhibitor itself. It is significant that nicotine, in concentrations sufficient to paralyze the ganglion cells, prevents the typical change in rhythm induced by the antiesterases. We were not able to elicit this change with acetylcholine itself, possibly due to the failure of this agent to gain access to the exact site at which the physiologically liberated acetylcholine acts.

The mechanism by which the anticholinesterase agents abolished the pendular movements in the rabbit gut and set up a slow peristaltic rhythm is not clear. The fact that the pendular activity can continue in the presence of atropine, procaine, thiamine, and nicotine suggests that this behaviour is independent of neurogenic factors. These drugs, however, readily prevent or abolish the peristaltic activity induced by the antiesterases, suggesting that this behaviour is on a neurogenic basis and depends on the accumulation of acetylcholine. It is conceivable that the onset of the peristaltic action, accompanied by strong constriction rings in the circular muscle, interferes with the contractions of the longitudinal muscle which constitute the pendular movements.

It was observed (table 2) that TEPP, physostigmine, and neostigmine were approximately equal in potency with respect to the minimal molar concentrations producing a change in rhythm in the isolated rabbit gut, while HETP and DFP were less effective. It is of interest to note that Koppanyi *et al.* (6), using a pharmacodynamic response in the dog to estimate the concentrations of TEPP, physostigmine, HETP, and DFP necessary to produce 50 per cent inhibition of cholinesterase arrived at the same relative potencies for these agents as were

derived from our experiments. Though our absolute concentrations were of the order of 100 times higher than those tabulated by Koppanyi *et al.*, they are approximately equivalent to the 50 per cent esterase inhibiting concentrations measured directly on rat brain and cockroach tissue (1) and considerably lower than similar measurements on human and horse serum (20). To what extent the isolated rabbit intestine might be useful in the bioassay of the cholinesterase inhibiting activity of chemical substances must be a subject of further investigation.

SUMMARY

1. The pharmacological effects of HETP and TEPP were compared with those of physostigmine, neostigmine and DFP on the blood pressure of anesthetized cats and dogs, on the electrocardiogram of anesthetized dogs, and on the isolated heart and intestine of the rabbit.

2. In these tests HETP and TEPP exhibited no qualitative differences in action from physostigmine and neostigmine.

3. With the exception of DFP, all of the drugs compared elicited qualitatively similar cardiovascular responses. DFP lacked a definite pressor effect and the onset of electrocardiogram changes was delayed.

4. Typical electrocardiographic changes following each drug were bradycardia, A-V block and dissociation, exaggeration and inversion of the T wave, and disappearance of the P wave.

5. The isolated rabbit heart was relatively insensitive to high perfusion concentrations or to single injections. Effective concentrations of each drug caused depression in amplitude without change in rate. The order of potency in this respect was DFP > HETP > TEPP > physostigmine.

6. The isolated rabbit intestine exhibited a high degree of sensitivity to all these agents. After a latent period the pendular movements were interrupted by a slow peristaltic type of activity. In their minimal effective concentrations these drugs bore the same relation to each other as in their reported anticholinesterase activities. Their effective concentrations were also of the same order as the 50 per cent cholinesterase inhibiting concentrations which have been reported.

7. These changes produced by HETP, TEPP, and DFP in the isolated intestine were not reversed by flushing the bath while the changes induced by physostigmine and neostigmine were reversed by flushing.

8. The tested pharmacologic actions of each of the five antiesterases were influenced in a similar way by other drugs, such as atropine, dibenamine, nicotine, procaine, and thiamine.

REFERENCES

1. DuBois, K. P., and Mangun, G. H.: *Proc. Soc. Exper. Biol. & Med.*, **64**: 137, 1947.
2. Hagan, E. C., and Woodard, G.: *Fed. Proc.*, **6**: 335, 1947.
3. Deichmann, W. B., and Witherup, S.: *Fed. Proc.*, **6**: 322, 1947.
4. Mangun, G. H., and DuBois, K. P.: *Fed. Proc.*, **6**: 353, 1947.
5. Roeder, K. D., and Kennedy, N. K.: *Fed. Proc.*, **6**: 191, 1947.

6. KOPPANYI, T., KARCZMAR, A. G., AND KING, T. O.: *Science*, **106**: 492, 1947.
7. BURGEN, A. S. V., KEELE, C. A., CHENNELL, M., DEL CASTILLO, J., FLOYD, W. F., SLOME, D., AND WRIGHT, S.: *Nature*, **160**: 760, 1947.
8. DAYRIT, C., MANRY, C. G., AND SEEVERS, M. H.: *THIS JOURNAL*, **92**: 173, 1948.
9. MENDEZ, R., AND RAVIN, A.: *THIS JOURNAL*, **72**: 80, 1941.
10. STEWART, G. N., AND ROGOFF, J. M.: *THIS JOURNAL*, **17**: 227, 1921.
11. ST. HEATHCOTE, R.: *THIS JOURNAL*, **44**: 95, 1932.
12. HAZARD, R.: *Presse Med.*, **53**: 149, 1945.
13. HAIMOVICI, H., AND PICK, E. P.: *Proc. Soc. Exper. Biol. & Med.*, **62**: 234, 1946.
14. UNNA, K., AND PICK, E. P.: *THIS JOURNAL*, **81**: 294, 1944.
15. GREENE, C. W., AND MANEVAL, K. E.: *THIS JOURNAL*, **46**: 303, 1932.
16. COHEN, S. L.: *Fed. Proc.*, **6**: 89, 1947.
17. JACKSON, E. E.: *Experimental Pharmacology and Materia Medica*, 2nd Edition, C. V. Mosby Co., 1939, p. 523.
18. UNNA, K., AND PICK, E. P.: *THIS JOURNAL*, **81**: 294, 1944.
19. DE ELIO, F. J.: *Brit. J. Pharmacol.*, **3**: 108, 1948.
20. MAZUR, A., AND BODANSKY, D.: *J. Biol. Chem.*, **163**: 261, 1946.
21. ADRIAN, E. D., FELDBERG, W., AND KILBY, B. A.: *Brit. J. Pharmacol.*, **2**: 56, 1947.
22. HAWKINS, R. D., AND MENDEL, B.: *Brit. J. Pharmacol.*, **2**: 173, 1947.
23. DIKSHIT, B. B.: *Quart. J. Exp. Physiol.*, **28**: 243, 1938.

TOXIC AND PATHOLOGIC EFFECTS OF XYLIDINE IN THE FASTING AND NON-FASTING STATES

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Although much is known of the toxicity of aromatic amino compounds, little is understood about factors which influence their effect *in vivo*. Claims have been made concerning the aggravating effect of alcohol on the methemoglobinemia of aromatic amines (1), and the protective effect of partial hepatectomy in aniline poisoning (2). In this report the advantage of the fasting over the postprandial state in withstanding exposure to vapors of commercial xylidine is demonstrated. Fasted cats were found to tolerate exposure to the xylidine-laden atmosphere for hours without evidence of serious disturbance. On the other hand, cats given large amounts of protein by forced feeding prior to exposure to xylidine exhibited a striking syndrome characterized by hyperpnea, panting, ptyalorrhea, agitation, and not infrequently death. Pathologic studies were made in search of a basis for this marked difference in reaction.

PROCEDURE. The procedure adopted for exposing animals to xylidine by inhalation resembled that previously reported from this laboratory (3). Aliquots of a single shipment of xylidine recently received or purified by distillation were placed in the bubbler, and air was passed through this and on into a 400-liter capacity exposure chamber at a rate of about 20.6 liters per minute. This vapor was diluted with air from a line having a flow of 170 liters per minute, thus insuring adequate ventilation. By employing a commercial preparation of the 6 xylidine isomers, conditions which might actually occur were more nearly reproduced. Xylidine concentration in the chamber atmosphere was determined by collecting an air sample in dilute H_2SO_4 , diazotizing and coupling with "H acid" (3).

Two to 6 cats, including at least one fasted and one yeast-fed animal (force-fed), were put in the chamber—usually in the evening—and were left there with intermittent observation for about 16 hours. Healthy adult cats were used which had been acclimatized to the laboratory environment at least four months. A few more female than male cats were used, but an equal sex distribution was achieved between the fasted and fed groups.

After the first three experiments the respiratory rate and estimated degree of salivation were recorded at each observation. Respiratory rates were counted in triplicate by gross inspection with a stop watch.

A suspension of dried brewer's yeast (Vita Food Green Label), 20 per cent by weight in skim milk, plus 5 per cent liver powder (Lilly) was given by gastric intubation in the first three experiments, whereas a 25 per cent suspension of yeast in skim milk was administered subsequently. The fasted animals were offered no food after their last regular meal, either 24 or 48 hours prior to exposure, and in the first three experiments were given water by intubation in amounts equal to the yeast-milk feedings.

In a first series of three experiments, it was planned to determine the effect of high protein intake and of p-aminobenzoic acid (PAB), a possible antidote (4), on survival, pathologic changes, methemoglobinemia, and Heinz body formation after exposure to xylidine vapors. Methemoglobin (MHb) and total hemoglobin concentration were determined according to the method of Horecker and Brackett (5). The blood turbidity ratio, which is

a quantitative photometric measure of the turbidity imparted to hemolyzed blood by the insoluble Heinz bodies, was measured by the method of Horecker (6). The force-fed animals were given 20 cc. per kgm. of yeast-liver-milk suspension three times during the 8 hours preceding exposure in all three experiments, and were given the same amount once, 24 hours before exposure in the first two experiments or tests. The xylidine concentration determined once during the exposure was 0.18 and 0.17 mgm. per liter in the first and second tests.

A second series of 8 experiments was carried out employing the same exposure procedure as previously except on two tests which were started in midforenoon. The yeast-milk suspension was tube-fed in 75 (\pm 25) cc. amounts once daily, 1 to 4 days before exposure and in 60 (\pm 10) cc. amounts once or twice in the 6 hours preceding the start of exposure. The xylidine concentration measured at different times on several runs varied between 0.11 and 0.27 mgm. per liter.

In a final series of 5 experiments the fed animals were given by intubation about 55 cc. of yeast-milk mixture or skim milk 2 to 4 hours and 50 cc. one-half to one hour before being exposed. One of the two fed animals in each of the last two tests received skim milk without yeast. In addition, the non-fasted cats were offered their regular diet of meat and milk after the first intubation. The xylidine concentration in the chamber atmosphere measured once during each test was $0.10 \pm .035$ mgm. per liter.

RESULTS. As noted in table I, all the fasted animals survived more than 24 hours after exposure, whereas 29 per cent of the fed animals died during exposure, and 17 per cent died the following day. Inasmuch as the effect of feeding on xylidine toxicity appeared to be an acute one, only the early survival data are given. Serious hepatic and other lesions developed later, equalizing the overall survival in the two groups.

The mean of the respiratory rates for all cats observed at specific intervals during, and in some instances before, exposure are shown in table II. The observation time varied in different tests, so the number of cats on which the mean was determined differs at each interval. The wide range of variation in the rates might be in part attributed to the tendency—referred to later—for the toxicity of xylidine, and the effect of feeding on this toxicity, to vary with the season.

A rate of 70 or more per minute was observed in 94 per cent of the fed animals and in only 22 per cent of the fasted cats. A rate of 200 or more per minute occurred in 59 per cent of the fed group but in only 4 per cent of the fasted animals. A respiratory rate greater than 200 was observed in 80 per cent of the animals in the second and third series that died within 24 hours of exposure. The accelerated respiration first appeared from 15 minutes to several hours after onset of exposure and generally wore off toward the end of exposure. The rate often decreased markedly shortly after an emesis.

The one animal exposed first in the fasted and months later in the fed condition developed tachypnea only on the latter occasion. Many of the fed animals, but none of the fasted, developed a syndrome characterized by marked agitation, panting, cyanosis, ptalorrhea and a glassy fixed expression about the eyes.

It was evident that the degree of ptalism resulting from the xylidine inhalation was greater in the fed animals. No salivation was noted in 74 per cent of the fasted animals in the second and third series, and only a moderate degree of ptalism was observed in the remaining 26 per cent. This contrasts with the 55

per cent of the fed animals showing marked to extreme ptyalism in which a steady flow of saliva dripped from the mouth and soaked the face, thorax, and forelegs.

TABLE I
Survival of fed and fasted cats exposed to xylidine inhalation

EXPERIMENTS	TOTAL NO. CATS	NO. DIED DURING EXPOSURE	NO. DIED WITHIN 24 HRS. AFTER END OF EXPOSURE	NO. SURVIVED > 24 HRS.
Fasted animals				
1- 3*	12	—	—	12
4-11†	17	—	—	14
12-15	10	—	—	10
Fed animals				
1- 3	6	5	1	0
4-11†	17	4	—	10
12-16‡	15	2	5	8

* Half the animals received PAB solution instead of water at intubation.

† A fasted and fed animal sacrificed for histopathology at end of exposure in three experiments.

‡ Survival the same in milk and milk + yeast-fed groups.

TABLE II
Respiratory rates in fed and fasted cats before and during xylidine exposure (Respirations per minute)

HR. FROM START OF EXPOSURE	FASTED ANIMALS			FED ANIMALS		
	No. of cats observed*	Mean respiratory rate	Std. dev.	No. of cats observed*	Mean respiratory rate	Std. dev.
Before exposure						
3- 6	8	43.0	16.5	8	41.4	16.1
0- 2	8	40.3	15.7	14	44.6	17.0
During exposure						
1/4	8	28.6	11.5	9	40.1	12.9
1- 2	7	31.3	14.0	12	157.5	72.5
3- 4	27	45.6	31.3	29	186.8	96.7
5- 6	11	53.3	60.0	13	151.1	69.2
7- 9	10	28.7	9.4	15	133.0	95.5
13-16	6	24.2	9.5	9	57.8	49.8

* When rates were recorded twice in a given time interval for an animal, the average of these two was used in computing the mean for all the cats.

MHb and Heinz body determinations on most of the animals exposed in the second series of experiments failed to reveal a difference between the fasted and the fed groups at the end of exposure. That the hyperpnea in fed cats was

not due to a high level of methemoglobinemia was shown by the virtual absence of MHb from blood samples of three fed animals taken at a time when they exhibited marked tachypnea. The effect of the administration of PAB to half the fasted animals in the first series of experiments which tended to show that PAB enhanced and prolonged the xylidine-induced rise in the MHb and Heinz body levels will be reported elsewhere.

Certain incidental observations may be mentioned. Periodic erythrocyte counts and hemoglobin and hematocrit determinations revealed severe anemia in a few cats about 7 days after exposure, followed in the survivors by a marked reticulocytosis sometimes reaching 70 per cent. In addition, numerous large Heinz bodies and a pink benzidine-positive plasma were observed in the anemic cats. Animals weak and prostrate in the cage two or three days after exposure were seen on occasion to produce a red-orange discoloration of the sawdust where contaminated with saliva. Reddening of wood shavings—a commonly observed property of urine from animals treated with aniline—indicates in this instance excretion of xylidine metabolic derivatives in the saliva.

The first and third series of experiments carried out in succeeding summers showed the difference between fasted and fed animals more strikingly than did the second series of 8 tests carried out during the intervening winter. However, the average xylidine concentration attained in the first and third series fell respectively above and below that found in the intervening (winter) series. This indicates a seasonal effect, the causes for which have not been investigated.

PATHOLOGY. Studies were made on formalin-fixed tissues of 27 fasted and 26 force-fed cats exposed to xylidine. Sections were stained with azure eosinate and frequently for hemosiderin, fat (7) and hemoglobin (8). Pathologic changes varied with the length of survival.

Changes in the Liver. In the first 48 hours, there was usually centrilobular congestion. Most of the liver cells, centrilobularly or diffusely, showed increased cytoplasmic eosinophilia and a variable amount of fat; a few cells were necrotic. Cats dying 3 to 11 days after exposure (3 to 11-day cats) often showed extensive centrilobular necrosis with disappearance of many liver cells, leaving a collapsed congested stroma moderately infiltrated chiefly by hemosiderin-laden and fat-laden phagocytes. Proliferation of small bile ducts and of bile duct epithelium was common. Most of these cats were jaundiced and showed inspissated bile casts in many canaliculi and bile-stained material in some bile ducts. One jaundiced cat dying 53 days after exposure showed in addition to such changes some interportal fibrous bands suggesting a possible beginning cirrhosis. Four other cats examined after the eleventh day showed no significant changes.

Changes in Other Organs. Pulmonary edema of variable extent was noted in 10 of 16 one- to five-day cats that had a maximum respiratory rate of 150 or more per minute during exposure and in only 1 (rate 84) of 11 such cats with a maximum respiratory rate below 150 (24 to 144). It was not seen after the fifth day. The myocardium of cats dying within 4 days after exposure showed slight to moderately marked, patchy or diffuse, fatty degeneration. The kidneys were often congested, and some showed hyaline and granular casts or bile-

stained material in a few tubules. Hemoglobin casts were noted in seven of nine 5-day and 6-day cats. The thymus of cats dying within 4 days after exposure was often markedly congested; about half showed scattered petechiae. No petechiae were noted after the fourth day, but involution of the thymus with cortical depletion of lymphocytes was common. The adrenal cortex of one 3-day cat showed several areas of hemorrhagic necrosis. The spleen of one 5-day cat showed several infarcts. Hemosiderosis of the spleen, liver, lungs and renal convoluted tubules was frequently marked after the third day.

DISCUSSION. Pulmonary edema noted here for the first time as a feature of xylidine toxicity was confined to, and involved the majority of, 1- to 5-day cats showing marked hyperpnea. Whether the edema constitutes a basis for the hyperpnea or simply occurs with it as a result of the same or separate etiologic processes remains a question. There is experimental evidence in rats that petechiae in the thymus may occur after severe anoxia (9) and rapid involution of the thymus may occur after a variety of noxious agents (10). Perhaps this may explain the changes in the thymus of our exposed cats.

It is thought that the severe anemia noted in a few cats was due to intravascular hemolysis since in these animals there was observed a pink, benzidine-positive plasma. Moreover, cats dying after the fourth day often showed hemoglobin casts in the renal tubules, brown benzidine-positive sediment in the urinary bladder, and hemosiderosis of the viscera.

The greater susceptibility of fed cats to acute effects of xylidine vapors cannot be attributed to a summation of toxic effects, for the milk, or yeast and milk feedings alone, produced no significant symptoms or lesions. Emesis occurring during exposure was often followed by considerable relief of respiratory distress. Symptoms in the fed animals subsided after exposure for several hours, so that the survivors in this group, aside from appearing weaker, resembled the controls at the end of exposure. The enteric absorptive process is apparently required for development of the effect.

As an explanation for the increased susceptibility during food absorption, it may be postulated that the postprandial acceleration in metabolism of amino acids—some of them aromatic amines—opens pathways whereby the oxidation, conjugation, or other metabolic alteration of xylidine to toxic intermediaries is augmented. Perhaps, also, the increased splanchnic blood and lymph flow in fed animals augments reabsorption of toxic derivatives excreted in the intestine (3). Finally, it is possible the increased circulatory and metabolic load in the postabsorptive state may overburden a toxic heart which is probably functionally impaired, as suggested by the fatty degeneration noted above.

SUMMARY

In contrast to fasted cats, those fed milk or milk and yeast reacted to xylidine vapors with an acute disturbance characterized by hyperpnea, panting, ptialism, and frequently early death. Survivors of both groups developed a high level of MHb and Heinz bodies in the blood, often followed by a severe hemolytic anemia and jaundice.

Pathologic studies showed pulmonary edema occurring chiefly in the hyperpneic animals and lesions of the liver, kidney, heart and thymus occurring in both groups.

REFERENCES

- | 1. HEUBNER, W., AND SCHWEDTKE, G.: Arch. f. Exp. Path. u. Pharmacol., 184: 80, 1937.
2. SCHEFF, G. J.: THIS JOURNAL, 70: 334, 1940.
- [3. VON OETTINGEN, W. F., et al.: Nat. Inst. Health Bull., 188, 1945.
4. SANDGROUND, J. H.: THIS JOURNAL, 78: 109, 1943.
5. HORECKER, B. L., AND BRACKETT, F. S.: J. Biol. Chem., 152: 669, 1943.
- | 6. HORECKER, B. L.: J. Indust. Hyg. and Toxicol., 27: 85, 1945.
7. LILLIE, R. D.: Histopathologic Technic. The Blakiston Company, Philadelphia, 1948.
8. DUNN, R. C., AND THOMPSON, E. C.: Arch. Path., 39: 49, 1945.
9. DALTON, A. J., JONES, B. F., PETERS, V. B., AND MITCHELL, E. R.: J. Nat. Cancer Inst., 6: 161, 1945.
10. SELYE, H.: Brit. J. Exp. Path., 17: 234, 1936.

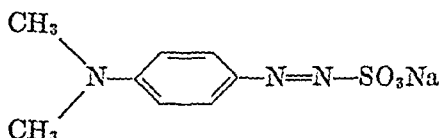
STUDIES ON THE TOXICITY AND PHARMACOLOGICAL ACTION OF p-DIMETHYLAMINO BENZENEDIAZO SODIUM SULFONATE (DAS)

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Among the interesting chemical compounds which came to the attention of the Technical Intelligence Committee at the end of the recent war in Germany was p-dimethylaminobenzenediazo sodium sulfonate (DAS) which has the following chemical structure:



This compound has been employed in Germany as a rodenticide; however, information concerning the toxicity of DAS, its efficacy as a rodenticide and its pharmacological action has been lacking in this country. Our interest in this new compound was stimulated not only by its possible value as a rodenticide but also by the close chemical similarity between DAS and the carcinogenic azo dyes.

The present investigation on DAS was carried out to (a) measure the acute toxicity to several species (b) ascertain its efficacy as a rodenticide and (c) observe the pathological and pharmacological effects produced by the compound. Some experiments were also performed on N,N-dimethyl-p-phenylenediamine since this compound was the starting product for the synthesis of DAS and might be formed during the metabolism of DAS as it is in the case of other similar azo dyes (1). Comparable experiments with DAS and N,N-dimethyl-p-phenylenediamine might, therefore, indicate whether the parent compound or a diamine derivative was responsible for the toxic action.

METHODS. Sprague-Dawley rats (ca. 200 grams), Carworth mice (ca. 20 grams), adult guinea pigs (ca. 600 grams), adult rabbits (ca. 3 kgm.) and adult dogs were employed for these studies. DAS was synthesized by diazotization of N,N-dimethyl-p-phenylenediamine and subsequent sulfonation according to the procedure of Stollé (2). Aqueous solutions of DAS and N,N-dimethyl-p-phenylenediamine were administered intraperitoneally and mortality was observed for 10 days following administration of the drug.

For blood studies on rats and guinea pigs samples were taken by cardiac puncture from ether-anesthetized animals before and at intervals after the administration of DAS or the

¹ The work described in this paper was done under contract between the Medical Division, Chemical Corps, U. S. Army and the University of Chicago Toxicity Laboratory. Under the terms of the contract the Chemical Corps neither restricts nor is responsible for the opinions or conclusions of the authors.

diamine. Blood samples were taken from the marginal ear vein of unanesthetized rabbits and by cardiac puncture from mice under sodium pentobarbital anesthesia (80 mgm./kgm. intraperitoneally). Successive blood samples were taken from the same animal except in the case of mice where only one sample of blood was withdrawn from each mouse.

Blood glucose was measured by the method of Folin and Malmros (3) using a Somogyi blood filtrate (4) and glycogen was determined according to the method of Good *et al.* (5). For the measurement of the reducing value of urine the samples of urine were diluted with an equal volume of 0.05 N oxalic acid, then thoroughly mixed with Fuller's Earth (150 mgm./cc.) and centrifuged (6). A Somogyi filtrate was prepared from the supernatant liquid and the reducing value was measured by the same procedure employed for the blood samples.

EXPERIMENTAL. *Toxicity of DAS and N,N-dimethyl-p-phenylenediamine to mammals.* The acute toxicity of DAS to mammals was measured in order to evaluate the rodenticidal action of the compound and to ascertain which species exhibited the greatest susceptibility toward the toxic agent. Comparable toxicity measurements were performed on N,N-dimethyl-p-phenylenediamine to observe whether the species susceptibility to this compound was similar to that for DAS since such a comparison might indicate whether the acute toxic action of DAS was due to the parent molecule or a diamine resulting from the degradation of DAS *in vivo* at the azo linkage. The acute toxicity of DAS and N,N-dimethyl-p-phenylenediamine given intraperitoneally to five species is shown by the data in table 1. The approximate LD₅₀ values for rats, mice and guinea pigs were determined by the log probability method while values for rabbits and dogs were obtained by gross inspection of the data.

These toxicity data demonstrate that there is considerable variation in the susceptibility of different species to DAS. In comparison with rats for which the LD₅₀ was 15 mgm./kgm., mice were over four times more resistant, guinea pigs twice as resistant and dogs and rabbits exhibited a susceptibility similar to that shown by rats. No sex or age differences in the susceptibility of animals to DAS were observed.

The species variation in susceptibility to N,N-dimethyl-p-phenylenediamine differed considerably from that observed with DAS. The LD₅₀ for mice and rats was nearly the same, being 21 and 25 mgm./kgm., respectively, while rabbits were about five times more resistant and guinea pigs twice as resistant as mice toward the compound. Thus, the difference in susceptibility of various species to the two compounds suggests that the toxic action of DAS does not depend upon its conversion to a diamine *in vivo*.

Symptomatology and pharmacodynamic observations of DAS. Acutely toxic doses of DAS produced a general depression and an apparent exhaustion of energy in all of the species studied. In rats after twice the LD₅₀ dose these reactions began within 2 hours after poisoning with the depression progressing until, in the terminal stages of poisoning, the animals became flaccid and made no attempt to right themselves when placed on their backs. Sometimes slight tremors were observed and convulsions almost always preceded death. Ordinarily death occurred within ten hours after acutely lethal doses of DAS but sometimes it was delayed for several days.

In dogs anesthetized with sodium pentobarbital (35 mgm./kgm. intraperitoneally) the blood pressure, as measured with a mercury manometer connected to a cannulated carotid artery, decreased after the intravenous administration of 30 mgm./kgm. of DAS. Five minutes after the injection of DAS the blood pressure began to decrease and by 35 minutes it had fallen to the extent of 45 mm. of mercury with no further decrease until death of the animals occurred. About 0.5 hour after DAS was administered the depth of respiration decreased, and the rate increased from 19 to 24/minute. The depth of respiration then increased and remained above normal for about 3 hours while the rate remained around 22/minute for 2 hours then gradually decreased. At 3 hours after DAS was administered the respiration became shallow and irregular and ceased in about 15 minutes at which time artificial respiration was begun. With continuous artificial respiration the blood pressure gradually dropped and the heart ceased beating about 20 minutes after the cessation of respiration.

TABLE 1

Toxicity of DAS and N,N-Dimethyl-p-phenylenediamine given intraperitoneally to mammals

SPECIES	DAS		N,N-DIMETHYL-p-PHENYLENEDIAMINE	
	No. of animals used	LD ₅₀ mgm./kgm.	No. of animals used	LD ₅₀ mgm./kgm.
Rats.....	74	15	47	21
Mice.....	70	70	89	25
Guinea pigs.....	36	30	32	45
Rabbits.....	12	10-20	8	100
Dogs.....	5	5-10	4	10-20

The symptoms which followed acute poisoning by N,N-dimethyl-p-phenylenediamine differed markedly from those produced by DAS. Within 10 to 30 minutes after the intraperitoneal administration of the compound animals developed tonic and clonic convulsions. Preceding the convulsions there existed a state of hyperexcitability which was soon followed by a series of convulsive seizures which terminated with death in about one hour after administration of the compound.

Pathological findings in rats after acute poisoning by DAS. As early as 15 to 30 minutes after the intraperitoneal administration of lethal doses of DAS to rats the yellow color of the compound was seen in the skin of the animals and was especially visible in the ears. The color was also observed in the urine as soon as 5 to 10 minutes after poisoning.

The gross pathological findings in rats acutely poisoned by DAS were not pronounced. Ordinarily the liver showed evidence of hyperemia of varying degrees of severity and pulmonary congestion was occasionally observed but pleural effusion was rarely noted. The kidneys of animals surviving for more than two days before succumbing to the poison were pale and swollen.

Microscopically the main pathological finding in rats 5 hours after the administration of 30 mgm./kgm. of DAS was a degeneration of the kidney tubules. Acute passive hyperemia of the liver was another consistent finding and occasionally a mild hyperemia could be detected in the mucosa of the gastrointestinal tract.

Acceptability of DAS to rats. To ascertain whether DAS was acceptable when offered to rats in the diet, feeding experiments were conducted. Diets containing 0.25 per cent, 0.5 per cent, and 1.0 per cent DAS were prepared by mixing the rodenticide with finely ground Purina Laboratory Chow. The diets were offered to unstarved rats overnight in place of their ordinary Purina Chow diet. For the five animals receiving 1 per cent DAS the average food consumption during the 12-hour period was 1.7 grams of food/kgm. and 4 of the 5 animals succumbed. When 0.5 per cent DAS was offered to 5 rats overnight the average food consumption was 4.9 grams/200-gram rat and all of the five animals died, and when 0.25 per cent DAS was offered the average food consumption was 4.9 grams/200-gram rat and three of the five animals died. These experiments indicated that DAS was effective and acceptable to rats when placed in the diet.

Because of the chemical similarity between DAS and the carcinogenic azo dyes, rats were fed ground Purina Laboratory Chow containing 0.10 per cent DAS to observe whether hepatomas would result. In animals which survived the feeding period hepatomas resembling those produced by dimethylaminoazobenzene appeared in approximately 12 months after the animals were placed on the diet. It is possible that the carcinogenic action might have become evident in a shorter period if synthetic diets optimum for carcinogenesis by azo dyes had been used.

To observe whether rats develop tolerance toward DAS after the administration of sublethal doses of the rodenticide five rats were given 10 mgm./kgm. of DAS intraperitoneally followed by a second injection of 20 mgm./kgm. three days later. All of the animals were dead within five hours after the second injection. Since the LD_{50} of DAS to rats is around 15 mgm./kgm. the inability of the animals to withstand a second dose of 20 mgm./kgm. indicated that little tolerance could have resulted from the first dose of the rodenticide.

The effects of DAS on blood glucose. In order to observe whether DAS interfered with carbohydrate metabolism blood glucose measurements were made on rats acutely poisoned by the rodenticide. The terminal convulsions produced by lethal doses of DAS suggested the possibility of a disturbance in carbohydrate metabolism. Blood glucose measurements were made before and at various intervals after the administration of the rodenticide. Rats, mice, guinea pigs and rabbits were employed to ascertain whether any species difference in the effects of DAS on carbohydrate metabolism could be observed.

The results of these measurements are shown in table 2 in which each value represents the average for at least four animals unless otherwise indicated by the number in parenthesis. It may be seen from these data that DAS caused hyperglycemia in all of the species studied with the degree of hyperglycemia

depending upon the dose of the drug and the species susceptibility to the toxic agent. Rats showed a relatively mild degree of hyperglycemia after the administration of 15 mgm./kgm. of DAS (LD_{50}) whereas twice the LD_{50} dose produced a marked hyperglycemia (172 mgm. per cent) within three hours after poisoning. Seven hours after poisoning the blood glucose fell to hypoglycemic levels and at the time of death it reached a very low level coinciding with the appearance of the terminal convulsions. The administration of 60 mgm./kgm. of DAS to rats caused a very rapid onset of hyperglycemia with a precipitous fall to hypoglycemic levels. Mice and guinea pigs showed similar changes but required a higher dose of the rodenticide to produce these changes, which corresponds to their greater resistance toward DAS. Rabbits, however, were quite resistant to the blood glucose changes produced by DAS and required approxi-

TABLE 2

Effect of DAS on the blood glucose of rats, mice, guinea pigs and rabbits

SPECIES	DOSE OF DAS	BLOOD GLUCOSE, MG. PER CENT						
		Hours after poisoning						
		Normal	1	3	5	7	10	At death
	mgm./ kgm.							
Rats	15	104	113	111	102	106		
	30	94	131	172	80	61		25
	60	99	205	62	45 (1)			
Mice	30	117			99			
	80	114						12
Guinea pigs	30	95	106	100	104			
	60	107	132	225	199		110	
Rabbits	30	90 (2)	91 (2)	97 (2)	97 (2)		86 (1)	
	60	82 (1)	227 (1)	dead				

mately four times the LD_{50} dose before hyperglycemia developed. Thus, DAS produced hyperglycemia in rats, rabbits, mice and rats with the extent and time of onset being dependent upon the dose of the drug and the species susceptibility to the rodenticide.

We were interested in comparing the effects of N,N-dimethyl-p-phenylenediamine on blood glucose with those produced by DAS. For these tests rats were poisoned with 25 mgm./kgm. of the diamine and blood samples were taken before the administration of the poison, at the onset of convulsions, and immediately preceding death. The average normal blood glucose level was found to be 89 mgm. per cent. At the beginning of convulsions, in from 5 to 12 minutes after poisoning, the average value was 100 mgm. per cent and just preceding death, in from 35 to 44 minutes after poisoning, it had risen to 125 mgm. per cent.

These results demonstrated that the diamine produced a slight increase in blood sugar but much less than that observed in DAS-poisoned animals.

Glycogen content of tissues from DAS-poisoned animals. The terminal hypoglycemia which was observed in DAS-poisoned animals suggested that the glycogen stores might be depleted. This was tested by measuring the liver glycogen of normal and DAS-poisoned animals which had been fasted for comparable periods of time. The results of glycogen measurements on rats, mice, and guinea pigs poisoned by DAS are shown in table 3 in which each value represents the average amount of glycogen for at least four animals.

These data show that acute poisoning by DAS results in a marked decrease in liver glycogen in the three species employed and that the degree of depletion depended upon the dose of the drug administered to a given species. There was also a correlation between the species susceptibility to DAS and the dose required to cause a fall in liver glycogen as evidenced by the observation that 30 mgm./kgm. of DAS nearly depleted the liver glycogen of rats while the same

TABLE 3
The effect of DAS on liver glycogen of rats, mice and guinea pigs

SPECIES	DAS mgm./kgm.	HOURS FASTED	PER CENT GLYCOGEN	
			Normal	5 Hours after poisoning
Rats	15	5	4.56	3.12
	30	5	2.14	0.114
Mice	30	5	0.79	0.74
	80	5	0.36	0.034
Guinea pigs	30	20	0.296	0.143
	60	20	0.296	0.132

dose had no significant effect on the liver glycogen of mice. Muscle glycogen of rats also decreased after DAS; a dose of 30 mgm./kgm. of DAS reduced the skeletal muscle glycogen of rats to one-half of the normal value. These studies on glycogen are in accord with the observations made on the blood glucose in which the response depended upon the dose of DAS and the species susceptibility to the rodenticide. They also give support to the possibility that the hypoglycemia is a result of the depletion of liver glycogen.

Antagonistic action of insulin and adrenal-demedullation toward the hyperglycemic action of DAS. To ascertain whether epinephrine was involved in the hyperglycemia and the depletion of liver glycogen by DAS, blood glucose and liver glycogen measurements were made on rats from which the adrenal medulla had been removed eleven days prior to use for the experiment. Similar measurements were also made on DAS-poisoned and normal rats treated with insulin. Two units of insulin/kgm. were injected subcutaneously one hour before the administration of DAS and a second injection was given three hours

rat/hour. These values indicate an increase in glucose and/or reducing substances in the urine after DAS poisoning but do not seem great enough to substantiate a marked decrease in tubular reabsorption of glucose during the sampling period.

Discussion. The present investigation has included a number of experiments designed to obtain information which would permit an evaluation of the rodenticidal action of p-dimethylaminobenzenediazo sodium sulfonate (DAS). Toxicity tests have shown that the LD₅₀ for DAS given intraperitoneally lies between 10 and 30 mgm./kgm. for albino rats, guinea pigs, rabbits and dogs. Thus, there was a relatively small variation in the susceptibility of these species to the rodenticide. On the other hand, mice were found to be considerably more resistant toward the compound. The oral LD₅₀ for albino rats was about 55 mgm./kgm. and it is, therefore, several times less toxic than sodium fluoroacetate (7) and alpha-naphthylthiourea (8). A marked difference in species susceptibility to DAS and N,N-dimethyl-p-phenylenediamine was observed which suggests that the compounds act differently and that the toxic action of DAS is not due to a diamine degradation product formed by cleavage at the azo linkage.

No significant age or sex difference in the susceptibility of rats to DAS was noted and the administration of a sublethal dose did not result in the acquisition of tolerance. The compound was acceptable to rats when offered at a concentration of 1 per cent or less in the diet. The main disadvantages attending the use of DAS as a rodenticide are its lower toxicity as compared with other new rodenticides and the absence of an effective antidote. Nevertheless, the substance may find practical use where the extremely high toxicity of fluoroacetate to many species (7) or the tolerance toward ANTU (8) limit the usefulness of these substances.

Because of the carcinogenic action of DAS the compound may be of value in future studies on the mechanism of carcinogenesis by azo dyes. Although it is a less potent carcinogen than several other azo dyes it presents an advantage in that it differs from the other carcinogenic azo dyes in being readily soluble in water and thus can be employed conveniently for *in vitro* experiments.

Studies on the blood glucose and tissue glycogen of DAS-poisoned animals have shown that this rodenticide produces a marked disturbance in carbohydrate metabolism. The hyperglycemia appeared to be induced by epinephrine since it could be prevented by adrenal-demodulation and insulin but these treatments did not affect the survival of DAS-poisoned animals. The terminal convulsions were due to hypoglycemia since they could be prevented or stopped by the administration of glucose. The hypoglycemia was undoubtedly the result of the depletion of tissue glycogen in animals poisoned by DAS, and the glycogen depletion seemed to be due principally to an inability of the tissues of DAS-poisoned rats to deposit glycogen. These results strongly suggest that DAS inhibits one or more of the enzymatic reactions involved in intermediary carbohydrate metabolism and future studies on the effects of DAS on enzyme systems may provide an explanation for the acute toxic action of the rodenticide.

SUMMARY

1. Measurement of the toxicity of p-dimethylaminobenzenediazo sodium sulfonate (DAS) administered intraperitoneally gave the following approximate LD_{50} values in mgm./kgm.: albino rats 15; albino mice 70, guinea pigs 30, rabbits 10-20, and dogs 5-10. The oral LD_{50} for albino rats was about 55 mgm./kgm.

2. No tolerance to DAS was observed in rats and the rodenticide was acceptable and produced high mortality of rats when placed in the diet at concentrations of 0.5 per cent and 1.0 per cent.

3. Toxicity measurements on N,N-dimethyl-p-phenylenediamine given intraperitoneally gave the following LD_{50} values in mgm./kgm.: albino rats 21, mice 25, guinea pigs 45, rabbits 100, and dogs 10-20. The differences in species susceptibility to this compound and DAS suggest that the latter substance does not undergo cleavage at the azo linkage before exerting its toxic effect.

4. DAS caused a generalized depression in all species, degeneration of the kidney tubules, hyperemia of the intestine and liver, a fall in blood pressure and respiratory paralysis.

5. Hyperglycemia followed by hypoglycemia occurred in animals poisoned by DAS. The extent of these changes was dependent upon the dose of DAS and the species susceptibility to the agent. The hyperglycemia could be prevented by insulin or by adrenal-demedullation but survival of the animals was not influenced by these treatments. The hypoglycemic convulsions could be terminated or prevented by glucose which prolonged the survival time but did not prevent ultimate death of the animals.

6. A depletion of liver and muscle glycogen of rats was observed after administration of DAS and the animals were unable to deposit liver glycogen from injected glucose; this indicated that DAS exerts an inhibitory action on glycogen synthesis.

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REFERENCES

1. STEVENSON, E. S., DOBRINER, K., AND RHOADS, C. P.: *Cancer Res.*, **2**: 160, 1942.
2. STOLLÉ, R.: *Berichte*, **45**: 2682, 1912.
3. FOLIN, O., AND MALMROS, H.: *J. Biol. Chem.*, **83**: 115, 1929.
4. SOMOGYI, M.: *J. Biol. Chem.*, **86**: 655, 1930.
5. GOOD, C. A., KRAMER, H., AND SOMOGYI, M.: *J. Biol. Chem.*, **100**: 484, 1933.
6. KOCH, F. C., AND HANKE, M. E.: *Practical Methods in Biochemistry*. 4th Ed., Williams & Wilkins, 246, 1943.
7. KALMBACH, E. R.: *Science*, **102**: 232, 1945.
8. RICHTER, C. P.: *J. A. M. A.*, **129**: 927, 1945.

THE EFFECT OF p-DIMETHYLAMINOBENZENEDIAZO SODIUM SULFONATE (DAS) ON THE ENZYMATIC REACTIONS OF INTERMEDIARY CARBOHYDRATE METABOLISM

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Evidence that acute poisoning by p-dimethylaminobenzenediazo sodium sulfonate (DAS) results in a disturbance of carbohydrate metabolism was obtained during an investigation of the toxicity and pharmacological action of this German rodenticide. This evidence, which is presented in the preceding communication, consisted of the observation that hyperglycemia followed by hypoglycemia and depletion of liver glycogen occurred after acute poisoning by DAS. The inability of fasted DAS-poisoned rats to deposit liver glycogen from injected glucose indicated that the toxic agent exerted an inhibitory action on glycogen synthesis. These findings stimulated our interest in investigating the action of DAS on the enzymatic reactions involved in intermediary carbohydrate metabolism in an effort to elucidate the mechanism of the acute toxic action of this azo compound in mammals.

The present communication contains the results of tests on the effect of DAS on several enzymatic reactions. In this study attempts were made to correlate *in vitro* findings with *in vivo* results in which the enzymatic reactions of tissues from DAS-poisoned animals were studied. Such attempted correlations are of considerable importance in explaining drug action on the basis of inhibition of enzymatic reactions. The results obtained in this study have demonstrated that DAS inhibits the oxygen consumption of liver slices and exerts a potent inhibitory action on the aerobic synthesis of high-energy phosphate compounds *in vitro* and *in vivo*. The inhibitory action of DAS on aerobic phosphorylation was dependent upon the dose of the rodenticide and upon the species susceptibility to the toxic compound.

METHODS. Sprague-Dawley rats (ca. 200 grams), Carworth mice (ca. 20 grams) and adult guinea pigs (ca. 600 grams) were employed for these studies. DAS was synthesized according to the procedure of Stollé (1) and aqueous solutions of the compound were administered by the intraperitoneal route.

Anaerobic glycolysis of brain tissue was measured according to the method of Elliott and Henry (2) and the oxidation of glucose by brain homogenates was measured by the procedure of Elliott *et al.* (3). The oxidation of several substrates by liver slices was measured using rat liver slices prepared according to the method of Deutsch (4). The liver slices were obtained from normal rats fasted for 16 hours prior to being sacrificed. Succinic de-

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hydrogenase and cytochrome oxidase activities were determined by the method of Schneider and Potter (5), malic dehydrogenase by the method of Potter (6) and adenosine triphosphatase by the procedure of DuBois and Potter (7).

The extraction, fractionation and measurement of the acid-soluble phosphorus compounds of rat tissues were carried out according to the procedure outlined by LePage and Umbreit (8). For these measurements the tissues were rapidly removed during sodium pentobarbital anesthesia (45 mgm./kgm.), and quickly frozen between two blocks of dry ice.

The effect of DAS on oxidative phosphorylation was ascertained by the method of Potter (9) using 0.3 cc. of a 10 per cent isotonic potassium chloride homogenate as the source of the enzymes, 26 mgm. of anhydrous creatine as the phosphate acceptor and succinate as the oxidizable substrate.

EXPERIMENTAL. *The action of DAS on the succinoxidase system.* The possibility that DAS might be metabolized *in vivo* to yield p-phenylenediamine or a methyl derivative of p-phenylenediamine, which compounds are known to be strong inhibitors of succinic dehydrogenase (10), stimulated our interest in investigating the effect of DAS on succinic dehydrogenase. *In vitro* and *in vivo* measurements of succinic dehydrogenase activity were, therefore, carried out on rat liver, kidney, lung and brain tissues. Various concentrations of DAS were tested by adding all of the components of the reaction mixture together with the inhibitor to the main compartment of the Warburg vessel and incubating for 20 minutes before the addition of the succinate from the side arm.

The effect of DAS on the succinoxidase activity of rat liver is shown in figure 1 from which it may be seen that DAS had an inhibitory action on this enzyme system *in vitro* but in all cases there was a lag period of about 20 minutes after the addition of the succinate before the inhibitory action of DAS became apparent. This was interpreted as indicating that some chemical change in the toxic agent took place to yield a product which inhibited the enzyme. It was noticed that the inhibition occurred after most of the yellow color due to DAS had disappeared from the reaction mixture which further suggested that a change in the DAS molecule occurred. As shown by Curves B and C of figure 1 final concentrations of $6.6 \times 10^{-5} M$ and $1 \times 10^{-4} M$ DAS respectively produced marked inhibition of enzyme activity when the inhibitor was incubated with the enzyme for 20 minutes before the addition of the substrate. Placing the inhibitor in the side-arm of the Warburg vessel with the succinate and adding both simultaneously decreased the amount of inhibition (Curve D, figure 1) as compared with the addition of the substrate after the inhibitor. This indicated a competition between succinate and the rodenticide for the enzyme. In contrast to the *in vitro* results no decrease in the succinoxidase activity of liver was observed when the livers were removed 5 hours after the administration of 30 mgm./kgm. of DAS.

DAS also produced an inhibition of the succinic dehydrogenase activity of kidney tissue *in vitro* after a preliminary lag period similar to that observed with liver. A final concentration of $1 \times 10^{-4} M$ DAS produced 2 per cent inhibition at 30 minutes, 24 per cent inhibition at 70 minutes and 67 per cent inhibition at

110 minutes after the addition of the succinate. As in the case of liver, however, no significant inhibition of the succinoxidase activity of kidney was observed 5 hours after the administration of 30 mgm./kgm. of DAS. A final concentration of $1 \times 10^{-4} M$ DAS produced no significant inhibition of the succinic dehydrogenase activity of brain and lung tissue *in vitro* nor was there any decrease in the activity of the enzyme in these tissues 5 hours after the administration of 30 mgm./kgm. of DAS. From these experiments it appeared that liver and kidney tissues are able to produce some change in DAS which results in the production of a succinic dehydrogenase inhibitor. In view of the inhibitory action of several phenylenediamines on succinic dehydrogenase which was observed by Potter and DuBois (10) it seems possible that the inhibitory

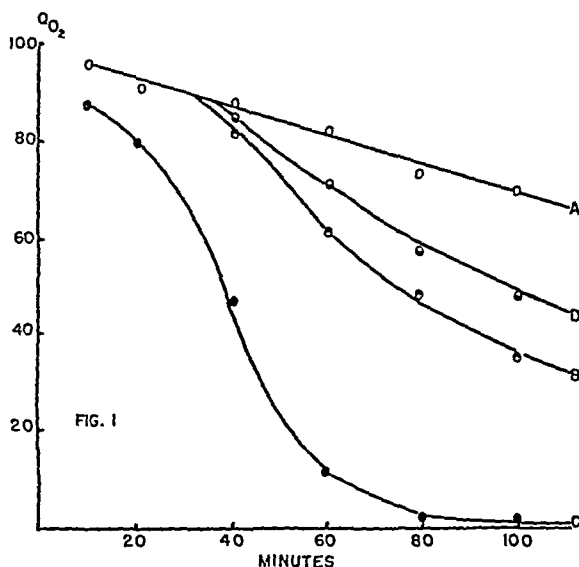


FIG. 1. THE EFFECT OF DAS ON THE SUCCINIC DEHYDROGENASE ACTIVITY OF RAT LIVER. Curve A, normal control; Curve B, $6.6 \times 10^{-5} M$ DAS added to the enzyme 20 minutes before the addition of the succinate; Curve C, $1 \times 10^{-4} M$ DAS added to the enzyme 20 minutes before the addition of the succinate; Curve D, $1 \times 10^{-4} M$ DAS added to the enzyme together with the succinate.

action of DAS on this enzyme system was due to cleavage of DAS at the azo linkage to form a toxic diamine. The lack of an inhibitory action of DAS on succinic dehydrogenase activity *in vivo* suggested that some other enzyme was more sensitive toward the inhibitor or that the enzyme-inhibitor complex was dissociated during homogenization and dilution of the tissue.

Because of the possibility that N,N-dimethyl-p-phenylenediamine might occur as a breakdown product of DAS the effect of this compound on the succinic dehydrogenase activity of rat liver was tested. Four rats were given 25 mgm./kgm. of the diamine intraperitoneally and sacrificed during convulsions which occurred one-half to three-fourths of an hour after poisoning. Whereas the QO_2 for the succinic dehydrogenase activity of normal livers was 82 that for

the livers of poisoned animals was 79. This compound had previously been shown (10) to produce 77 per cent inhibition of succinic dehydrogenase at a final concentration of $1 \times 10^{-5} M$ *in vitro*. DAS and N,N-dimethyl-p-phenylenediamine therefore act similarly on succinic dehydrogenase inhibiting the action of the enzyme *in vitro* but not *in vivo*.

Since the succinoxidase system (5) employed for these experiments requires the catalytic action of succinic dehydrogenase, cytochrome *c*, and cytochrome oxidase it was necessary to investigate the action of the toxic substance on the latter two catalysts before attributing its inhibitory effect solely to an interaction with succinic dehydrogenase. It was found that a final concentration of $2 \times 10^{-4} M$ DAS had no effect on cytochrome oxidase activity of rat liver while the same concentration had produced complete inhibition of the succinoxidase system. Variation in the cytochrome *c* content of the succinoxidase system did not alter the per cent inhibition by DAS; therefore, DAS was not reacting with this component of the system and its inhibitory effect could be attributed to an action on succinic dehydrogenase.

The absence of an inhibitory effect by DAS on the malic dehydrogenase system. To test the effect of DAS on a coenzyme I-linked dehydrogenase the malic dehydrogenase system was employed. Whereas the normal QO_2 value for malic dehydrogenase of liver was 54 the addition of DAS at final molar concentrations of 1×10^{-3} and 1×10^{-4} resulted in QO_2 values of 48 and 53, respectively. DAS, therefore, produced no significant inhibition of malic dehydrogenase *in vitro*.

Using the malic dehydrogenase test system with fumarate in place of malate as the substrate it was possible to obtain an indication of whether DAS inhibits fumarase activity. The use of fumarate in place of malate as the substrate requires the catalytic action of fumarase to convert fumarate to malate which is then oxidized by the malic dehydrogenase system. The absence of an inhibitory effect by DAS at $1 \times 10^{-3} M$ final concentration indicated that the rodenticide did not inhibit the activity of fumarase.

The influence of DAS on anaerobic glycolysis and the oxidation of glucose by brain and liver. In initiating studies on the effect of DAS on glycolysis and respiration isotonic brain homogenates and liver slices were employed. An inhibitory action by DAS on the overall processes of either glycolysis or respiration could then be followed by an examination of the action of the toxic compound on individual enzymatic reactions in an attempt to locate the specific site of action.

DAS exerted no effect on the rate of anaerobic glycolysis of rat brain homogenates and liver slices. In the case of brain the normal CO_2/N_2 was 8.2 and in the presence of final concentrations of $1 \times 10^{-2} M$ and $1 \times 10^{-3} M$ DAS the values were 8.0 and 7.5, respectively. Similarly, DAS had no effect on the rate of anaerobic glycolysis of liver slices. Whereas the normal QCO_2/N_2 value was 6.3 for rat liver slices the presence of a final concentration of $1 \times 10^{-4} M$ DAS resulted in a value of 6.1. These experiments indicated that DAS in the concentrations employed had no significant effect on anaerobic glycolysis which seemed to eliminate inhibition of glycolytic enzymes from further consideration

in the search for the cause of the disturbance in carbohydrate metabolism produced by DAS.

The oxidation of glucose by rat brain homogenates was inhibited *in vitro* by DAS. Final concentrations of $1 \times 10^{-3} M$, $1 \times 10^{-4} M$ and $1 \times 10^{-5} M$ DAS produced 89, 63 and 40 per cent inhibition, respectively, of the oxidation of glucose. However, no depression of the rate of oxidation of glucose by brain tissue of rats given 30 mgm./kgm. of DAS could be detected when the animals were sacrificed 5 hours after administration of the compound.

DAS also inhibited the oxygen consumption of liver slices suspended in Krebs-Ringer phosphate solution (11) buffered at pH 7.4 and containing 0.02 *M* glucose. The presence of a final concentration of $1 \times 10^{-4} M$ DAS depressed the QO_2 from a normal value of 7.0 to 3.8. An inhibitory action by DAS on the cellular respiration of liver slices *in vivo* also occurred. Livers removed from rats 5 hours after the administration of 30 mgm./kgm. of DAS gave an average QO_2 value 5.6 representing a 21 per cent decrease in oxygen consumption. The inhibitory action of DAS on cellular respiration suggested that further experiments should be directed toward studying the action of the rodenticide on the various steps of the oxidative phase of carbohydrate metabolism.

The inhibitory action of DAS on the oxidation of pyruvate and citrate by rat liver slices. The lack of an inhibitory action by DAS on the oxidation of succinate, malate and fumarate indicated that the depressant action of the toxic compound occurred at some other step in the oxidative phase of carbohydrate metabolism. The effect of DAS on the respiration of liver slices was therefore tested employing several of the intermediates of the Krebs citric acid cycle as substrates. The substrates employed for these experiments were fumarate, malate, succinate, citrate, pyruvate and oxalacetate. L-glutamate was also included in these studies.

The substrates were prepared as 0.3 *M* solutions with the exception of oxalacetate which was prepared as a 0.15 *M* solution. Two-tenths cc. of the substrate was added to enough Krebs-Ringer phosphate medium to give a final volume of 3.0 cc. in the Warburg vessel. Where DAS was tested *in vitro* 0.3 cc. of a solution ($1 \times 10^{-3} M$) replaced an equivalent amount of Krebs-Ringer phosphate giving a final inhibitor concentration of $1 \times 10^{-4} M$.

For testing the effect of DAS on the oxidation of various substrates by liver slices one sample was included without the addition of a substrate to measure the endogenous respiration and another sample with an added substrate but without DAS was included to measure the stimulation of respiration due to the substrate. A third sample contained DAS and no added substrate to measure the effect of the compound on the endogenous respiration and another sample contained DAS and an added substrate to note the effect of DAS on the oxidation of the particular substrate tested. The results of these tests are shown in table 1 in which each value is the average of at least 3 determinations.

As may be seen from the data in table 1 the stimulation of the respiration of liver slices produced by malate, succinate and oxalacetate was unaffected by the addition of DAS. However, the additional oxygen consumption due to fumarate, pyruvate and citrate was decreased by the presence of $1 \times 10^{-4} M$ DAS.

To ascertain whether the inhibition of the utilization of these oxidative substrates also occurred in DAS-poisoned animals *in vivo* tests were performed on the livers of rats poisoned 5 hours previously with 30 mgm./kgm. of DAS.

TABLE 1

Effect of DAS in vitro and in vivo on the oxidation of several substrates by rat liver slices

SUBSTRATE	QO ₂		
	Control	1×10^{-4} M DAS	In vivo 5 hours after 30 mgm./kgm. DAS
Fumarate.....	9.3	4.9	8.1
No fumarate.....	7.0	4.2	5.7
Stimulation.....	2.3	0.7	2.4
Malate.....	8.0	4.9	7.3
No malate.....	7.0	3.9	5.6
Stimulation.....	1.0	1.0	1.7
Succinate.....	15.9	11.4	14.8
No succinate.....	7.1	3.0	5.7
Stimulation.....	8.8	8.4	9.1
Citrate.....	10.5	4.0	7.8
No citrate.....	7.2	3.3	5.4
Stimulation.....	3.3	0.7	2.4
Pyruvate.....	10.1	5.1	7.3
No pyruvate.....	6.8	3.8	5.6
Stimulation.....	3.3	1.3	1.7
Oxalacetate.....	9.2	6.7	
No oxalacetate.....	6.9	4.2	
Stimulation.....	2.3	2.5	
1-Glutamate.....	8.4		7.7
No 1-glutamate.....	6.7		5.5
Stimulation.....	1.7		2.2

The results of these tests are also shown in table 1 where it may be seen that the stimulation of respiration by malate, succinate, glutamate and fumarate was not decreased when the livers of poisoned rats were used in place of the livers of normal animals. The additional oxygen consumption due to pyruvate and citrate, however, was depressed in the liver samples from poisoned animals.

These findings are in agreement with results obtained on the isolated succinoxidase and malic dehydrogenase systems in which DAS did not exert an inhibitory action *in vivo*. The present experiments indicate that the inhibitory action of DAS on the oxidative phase of carbohydrate metabolism might be explained on the basis of inhibition of the oxidation of citrate and pyruvate.

The influence of DAS on the concentration of acid-soluble phosphorus compounds of rat liver and kidney. Although DAS had no direct inhibitory action on glycolysis the depression of the cellular respiration by the compound might lead to changes in the concentration of some of the phosphorylated intermediates of glycolysis. The dependence of the synthesis of high-energy phosphate bonds upon oxidative reactions made the possibility of depletion of such compounds by DAS seem probable. The concentrations of acid-soluble phosphorus compounds

TABLE 2

The distribution of acid-soluble phosphorus compounds in the liver and kidney of normal and DAS-poisoned rats

COMPOUND	LIVER		KIDNEY	
	Normal	5 hours after 30 mgm./kgm. DAS	Normal	5 hours after 30 mgm./kgm. DAS
(Micromoles per 100 grams of wet tissue)				
Phosphocreatine.....	406	0	22	0
Adenosine triphosphate.....	74	96	0	0
Adenosine diphosphate.....	245	207	0	0
Adenylic acid.....	83	88	143	130
Glucose-1-phosphate.....	136	85	163	131
Glucose-6-phosphate.....	554	675	454	575
Fructose-6-phosphate.....	19	20	25	25
Fructose-1,6-diphosphate.....	67	20	30	24
Triose phosphate.....	18	30	0	0
Phosphopyruvate.....	15	37	54	73
Coenzymes.....	9	9	18	17
Inorganic phosphorus.....	235	806	872	885
Total phosphorus.....	2670	2840	3025	2425

in rat tissues were, therefore, measured after the administration of 30 mgm./kgm. of DAS given intraperitoneally. The liver was chosen for these studies because DAS had been shown to produce biochemical changes in this organ as evidenced by depletion of liver glycogen and inhibition of cellular respiration. Kidney tissue was also included in these measurements because of the evidence that a degeneration of the kidney tubules occurs in rats poisoned by DAS. Four normal unfasted male rats and 4 rats sacrificed 5 hours after poisoning were employed for the measurements.

The results of these determinations are summarized in table 2 in which each value is the average for 4 rats. The data indicate that DAS produces changes in the distribution of the acid-soluble compounds of both liver and kidney of rats. The most pronounced change was a depletion of phosphocreatine of both liver and kidney tissue. The decrease in phosphocreatine partly accounts for

the increase in inorganic phosphorus in the tissues of poisoned animals. There was also a slight decrease in glucose-1-phosphate and a small increase in glucose-6-phosphate as well as small increases in phosphopyruvate and triose phosphate.

In order to ascertain whether the phosphocreatine of tissues other than liver and kidney was depleted in DAS-poisoned animals measurements were made on heart and skeletal muscle of rats taken 5 hours after the intraperitoneal administration of 30 mgm./kgm. of DAS. It was found that DAS had no effect on the phosphocreatine level of these tissues in contrast to the effects on liver and kidney phosphocreatine.

The inhibitory effect of DAS on oxidative phosphorylation by rat, mouse and guinea pig homogenates. The depletion of phosphocreatine in liver and kidney tissue from DAS-poisoned rats suggested the possibility that the toxic agent inhibited the esterification of phosphate. Attention was drawn to this possi-

TABLE 3

The effect of DAS on oxidative phosphorylation by rat, mouse and guinea pig kidney homogenates in vitro

SPECIES	MOLAR CONCENTRATION OF DAS	E (MICROGRAMS OF P ESTERIFIED PER 30 MGM. WET TISSUE)	MM. O ₂ CONSUMED PER 10 MINUTES	% INHIBITION
Rats	Control	136	39	—
	1×10^{-4}	53	36	61
	1×10^{-5}	75	41	45
	5×10^{-6}	83	39	39
	1×10^{-6}	137	58	0
Mice	Control	115	36	—
	1×10^{-4}	30	36	74
Guinea pigs	Control	148	34	—
	1×10^{-4}	104	40	30
	1×10^{-5}	144	44	2

bility not only by the depletion of phosphocreatine but also by the inhibitory action of DAS on cellular respiration. The recent development of a method by Potter (9) for measuring coupled oxidation and phosphorylation has made it possible to obtain quantitative measurements of the effect of drugs *in vitro* and *in vivo* on this important synthetic reaction. It was thus possible to ascertain the effect of DAS on phosphocreatine synthesis.

For our measurements 0.3 cc. of a 10 per cent kidney homogenate prepared in isotonic KCl was employed as the source of the enzymes, succinate as the oxidizable substrate and creatine as the phosphate acceptor. *In vitro* measurements were carried out on kidney tissue of normal animals employing various concentrations of DAS and *in vivo* measurements were performed on kidney tissue taken from poisoned animals. The effects of various concentrations of DAS on aerobic phosphorylation *in vitro* are shown in table 3 in which E represents the micrograms of inorganic phosphate esterified by 30 mgm. of wet tissue in 20 minutes

and O_2 represents the cmm. of oxygen consumed by the tissue during the same period of time (9).

The results of these tests demonstrated that DAS is a strong inhibitor of aerobic phosphorylation *in vitro*. At the low concentration of $5 \times 10^{-6} M$ 39 per cent inhibition of transphosphorylation by rat kidney homogenates was observed without a significant effect on oxygen consumption. This indicated that the transphosphorylating enzyme was inhibited rather than the oxidation of succinate; thus the rodenticide essentially uncoupled phosphorylation and oxidation. The reaction was also inhibited when guinea pig and mouse kidneys were used as the source of the enzyme. However, in spite of the marked difference in the susceptibility of rats, mice and guinea pigs toward the acute toxic action of

TABLE 4

The effect of DAS on oxidative phosphorylation by rat, mouse and guinea pig kidney tissue in vivo

SPECIES	DOSE OF DAS	HOURS AFTER ADMINISTRATION DAS	E (MICROGRAMS OF P ESTERIFIED PER 30 MCM. WET TISSUE)	CMM. O_2 CONSUMED PER 10 MINS.	% INHIBITION
	<i>mgm./kgm.</i>				
Rats	normal	—	136	39	—
	15	5	32	37	76
	30	1	71	39	48
	30	5	—75	34	100
Mice	normal	—	115	36	—
	30	5	114	40	0
	90	5	17	43	85
Guinea pigs	normal	—	148	34	—
	30	5	102	31	31
	60	5	80	32	46

DAS there was no marked difference in the amount of DAS necessary to inhibit phosphorylation by the kidney tissue from the three species *in vitro*.

To ascertain the effect of DAS on aerobic phosphorylation *in vivo* rats, mice and guinea pigs were given various doses of the compound and the kidneys were removed 5 hours later for aerobic phosphorylation measurements. The results of these tests are shown in table 4 in which each value is the average for at least 4 animals. It may be seen from these data that DAS exerted a marked inhibitory action on phosphocreatine synthesis *in vivo* with the extent of inhibition being dependent upon the dose of the compound administered. Furthermore, there was a correlation between the species susceptibility to DAS and the dose required to inhibit the transphosphorylation system *in vivo*. The LD_{50} values for rats, guinea pigs and mice are 15, 30, 70 mgm./kgm. of DAS, respectively. While 30 mgm./kgm. of DAS produced complete inhibition of aerobic phosphorylation by kidney tissue of rats, no inhibition of the reaction in the

kidneys of mice and only 31 per cent inhibition occurred in guinea pig kidneys after this dose of DAS.

In all of the tests performed it was observed that the phosphorylation of creatine was inhibited without inhibition of the oxidation of succinate. This was not unexpected since experiments described above on succinic dehydrogenase demonstrated that the inhibition of this enzyme by DAS occurred only after a lag period of about 40 minutes from the time of addition of the inhibitor to the enzyme and since the phosphorylation experiments were performed in 20 minutes inhibition of succinic dehydrogenase would not be manifested during this period. These experiments indicate that DAS produces its inhibitory effect on some enzyme or enzymes directly involved in the transphosphorylation process.

It has recently been reported by Loomis and Lipmann (11) that dinitrophenol reversibly uncouples phosphorylation from oxidation. It was of interest to observe whether the inhibitory action of DAS on oxidative phosphorylation was also reversible. For these experiments a 20 per cent kidney homogenate was

TABLE 5

The reversibility of the inhibitory action of DAS on aerobic phosphorylation

TREATMENT	E (MICROGRAMS OF P ESTERIFIED PER 60 MG.M. WET TISSUE)	MM. O ₂ CONSUMED PER 10 MINS.
Unwashed enzyme preparation		
Control.....	102	39
1×10^{-4} M DAS added.....	-7	48
Washed enzyme preparation		
Control.....	76	48
1×10^{-4} M DAS added before washing.....	78	47
1×10^{-4} M DAS added after washing.....	-18	61

prepared in isotonic potassium chloride and divided into 3 portions one of which served as the control and received no further treatment. To a 3 cc. portion of the homogenate DAS was added to give a final concentration of 1×10^{-4} M and the mixture was allowed to stand for 30 minutes after which time it was centrifuged at approximately 5000 rpm in a refrigerated centrifuge for 30 minutes. The supernatant liquid was then discarded and the precipitate was well mixed with 3 cc. of isotonic potassium chloride and again centrifuged. After 3 such washings the precipitate was taken up in 3 cc. of potassium chloride solution and employed for measurements of oxidative phosphorylation. Another portion of the kidney homogenate without the addition of DAS was carried through the same procedure and served as a control to indicate the amount of loss of the enzyme during the washing procedure. In no instance was the temperature of the homogenates allowed to exceed 5°C. The phosphorylative activities of the samples are shown in table 5 in which it may be seen that the inhibitory action of DAS was reversible under the conditions of this experiment. While the washing procedure resulted in 26 per cent loss of phosphorylative activity of the homogenate the addition of DAS before washing the enzyme resulted in no

further loss of activity. This demonstrated that the inhibitor could be removed by washing the enzyme. That 1×10^{-4} M DAS would have resulted in complete inhibition of phosphorylation by this enzyme preparation if it were not removed by washing was adequately demonstrated by complete inhibition of the reaction after addition of DAS to the washed and the untreated kidney homogenate.

DISCUSSION. An investigation of the effects of p-dimethylaminobenzenediazo sodium sulfonate (DAS) on intermediary carbohydrate metabolism has shown that the compound has no direct inhibitory action on glycolysis but does exert an inhibitory effect on the oxidative phase of carbohydrate metabolism. In attempts to ascertain the exact site of action of DAS on the oxidation of carbohydrate several *in vitro* and *in vivo* tests were performed. DAS inhibited succinic dehydrogenase *in vitro* but the inhibition occurred only after a lag period during which time it appeared that the compound had undergone a chemical change. It is likely that cleavage at the azo linkage occurred to yield a diamine which then inhibited succinic dehydrogenase. The absence of an inhibitory effect by DAS on succinic dehydrogenase *in vivo* and on the oxidation of succinate by liver slices indicated that this enzyme was not involved in the acute toxic action of this azo compound. Similarly cytochrome oxidase, malic dehydrogenase and fumarase were eliminated from further consideration in the mechanism of the toxic action of DAS. The observation that the oxidation of pyruvate and citrate by liver slices was inhibited by DAS *in vitro* and *in vivo* indicated that DAS inhibits one or more of the biocatalysts involved in the formation of alpha-ketoglutarate from pyruvate. The action of DAS on the oxidation of carbohydrate can be explained, at least partially, on the basis of inhibition of this portion of the dicarboxylic acid cycle. This inhibitory effect may also be partially responsible for the inhibition of glycogen synthesis by DAS.

We attached considerable significance to the depletion of phosphocreatine in some of the tissues of DAS-poisoned rats. Phosphocreatine is generally assumed to constitute a store of energy-rich phosphate which together with adenosine triphosphate functions in the transfer of chemical energy into energy available for the performance of tissue functions. Since DAS had no direct effect on glycolysis we reasoned that the depletion of phosphocreatine might result from an inhibition of the aerobic phosphorylation of creatine by DAS. It was found that DAS had a potent inhibitory effect on the aerobic synthesis of phosphocreatine. This effect, however, was not due to inhibition of oxygen consumption but rather to a direct action on the transphosphorylating mechanism since no depression of oxygen consumption occurred in the test system employed for phosphorylation. Thus, DAS, like dinitrophenol (12), uncoupled oxidation from phosphate esterification.

In connection with the mechanism of acute poisoning by DAS greater significance is attached to the inhibition of phosphorylation than to any of the other effects produced by the compound on the enzymes studied. DAS inhibited aerobic phosphorylation both *in vivo* and *in vitro* and there was a correlation between the species susceptibility to acute poisoning by DAS and the dose

of the compound necessary to inhibit aerobic phosphorylation *in vivo*. *In vitro*, however, the kidney tissue of rats, mice and guinea pigs were of similar susceptibility to the inhibitory action of DAS on aerobic phosphorylation. These differences between the *in vitro* and *in vivo* effects of DAS suggest that a difference in the rate of detoxification in the various species is responsible for the variation in susceptibility to the toxic agent rather than variations in the concentrations of sensitive enzymes.

The strong inhibitory action of DAS on phosphate esterification *in vivo* and *in vitro* gives support to the possibility that some of the acute toxic effects of this compound may result from inhibition of this important biochemical reaction. In support of such a possibility was the finding that the tissues which showed biochemical and pathological changes after DAS, namely the liver and kidney, are the same tissues in which DAS produced a depletion of phosphocreatine.

SUMMARY

1. *In vitro* studies demonstrated that dimethylaminobenzenediazo sodium sulfonate (DAS) inhibits the succinic dehydrogenase activity of rat liver and kidney after a preliminary incubation of the enzyme with the rodenticide. However, no inhibition of the succinic dehydrogenase activity of the tissues of rats was observed at 5 hours after the administration of 30 mgm./kgm. of DAS. No inhibitory effect by DAS was observed on cytochrome oxidase, malic dehydrogenase and fumarase.

2. The oxidation of glucose by rat brain homogenates was inhibited *in vitro* by concentrations of DAS above $1 \times 10^{-6} M$; however, no depression of glucose oxidation by brain tissue from DAS-poisoned rats was observed. The respiration of liver tissue was inhibited *in vitro* and *in vivo* by DAS. The toxic agent had no effect on the rate of anaerobic glycolysis by rat liver slices and brain homogenates.

3. DAS had no effect on the oxidation of fumarate, succinate, malate and oxalacetate by liver slices taken from rats 5 hours after the administration of 30 mgm./kgm. of the toxic compound. However, the stimulation of respiration of liver slices by pyruvate and citrate was depressed both *in vitro* and *in vivo* by DAS.

4. The phosphocreatine of rat liver and kidney tissue was depleted within 5 hours after the administration of 30 mgm./kgm. of DAS while the phosphocreatine of skeletal muscle and cardiac muscle was unaffected by this dose of the compound. Other changes in the distribution of acid-soluble phosphorus compounds in the liver and kidney of DAS-poisoned rats consisted of a slight decrease in glucose-1-phosphate and a small increase in glucose-6-phosphate, phosphopyruvate and triose phosphate.

5. DAS exerted a marked inhibitory effect on the aerobic phosphorylation of creatine by rat, mouse and guinea pig kidney homogenates. Aerobic phosphorylation was also inhibited in the tissues of animals given lethal doses of DAS. The inhibition *in vivo* was dependent upon the dose of the compound administered and varied with the species susceptibility to DAS.

REFERENCES

1. STOLLÉ, R.: *Berichte*, 45: 2682, 1912.
2. ELLIOTT, K. A. C., AND HENRY, M.: *J. Biol. Chem.*, 163: 361, 1946.
3. ELLIOTT, K. A. C., AND HENRY, M.: *J. Biol. Chem.*, 163: 351, 1946.
4. DEUTSCH, W.: *J. Physiol.*, 87: 560, 1936.
5. SCHNEIDER, W. C., AND POTTER, V. R.: *J. Biol. Chem.*, 149: 217, 1943.
6. POTTER, V. R.: *J. Biol. Chem.*, 165: 311, 1946.
7. DuBois, K. P., AND POTTER, V. R.: *J. Biol. Chem.*, 150: 185, 1943.
8. UMBREIT, W. W., BURRIS, R. H., AND STAUFFER, J. F.: *Manometric Techniques and Related Methods for the Study of Tissue Metabolism*. 2nd printing, Burgess Publishing Co., Minneapolis, Minn., p. 160.
9. POTTER, V. R.: *J. Biol. Chem.*, 169: 17, 1947.
10. POTTER, V. R., AND DuBois, K. P.: *J. Gen. Physiol.*, 26: 391, 1943.
11. LOOMIS, W. F., AND LIPMANN, F.: *J. Biol. Chem.*, 173: 807, 1948.



John Auer

1875-1948

It is with profound sorrow that we record the death on April 30, 1948 of Dr. John Auer from a cardiac attack, at St. Mary's Hospital, St. Louis, Missouri, which he had entered about a week previously for a physical examination. Dr. Auer's passing removes from the Society for Pharmacology and Experimental Therapeutics one of its active organizers and distinguished members who exerted a strong influence in the upbuilding of the Society and in the promotion and application of the science of American pharmacology.

Dr. Auer was born in Rochester, New York, on March 30, 1875. His college preparation for his field was obtained at the Universities of Michigan and Johns Hopkins; he received his B.S. from the former in 1898 and his M.D. from the latter in 1902. After finishing his formal course in medicine, he served for a year as House Officer at the Johns Hopkins Hospital, following which he became a Fellow in the Rockefeller Institute for Medical Research, continuing there as Assistant, Associate, and Associate Member until 1921. During the years 1906-7 he was sent to the Harvard Medical School for special training in physiology, where he also served as Instructor in Physiology. In 1921 he accepted the Professorship of Pharmacology in the St. Louis University School of Medicine and was its Departmental Chairman until his death. From 1924 he also served continuously as Pharmacologist to the St. Mary's Hospital, of St. Louis. During the first World War he joined the Reserve Corps of the Army, in which service he attained the rank of Major.

Dr. Auer contributed much to both physiological and pharmacological sciences. Some of his studies at the Rockefeller Institute were done in conjunction with the late Samuel J. Meltzer. His scientific papers were of a varied nature and number about 150, among which were investigations on the heart, the kidney, the liver, the gall bladder, connective tissue, digestion, respiration, reflexes, functional disturbances caused by anaphylaxis, tetanus, war gases, and the physiological action of various drugs. During his war service he was the first to use magnesium sulfate intravenously as a relaxant in a case of tetanus.

In addition to being an organizer of the Society for Pharmacology and Experimental Therapeutics, he was its secretary from 1912 to 1917 and its president from 1924 to 1927. He was also a member of the Association of American Physicians, Society for Experimental Biology and Medicine (being its vice president from 1917-18), the American Physiological Society, American Association for the Advancement of Science, the Harvey Society, the St. Louis Academy of Sciences and the St. Louis Medical Society. A few weeks before his death he received honorary membership in the St. Louis Society of Anesthesiologists "for his pioneering work in the field of anesthesiology" and because his "continued interest has proved a source of stimulation to the younger members in this field and has contributed greatly to this branch of medical science."

Dr. Auer was the embodiment of the true investigator whose imaginative insight, perseverance and powers of analysis and synthesis were of a remarkably high order, his devotion to truth uncompromising. These fine qualities he brought to his students, together with his unselfishness and a vast knowledge in related fields, thus developing a breadth of view and an intelligence that is essential for the scientific physician. As a tribute to his outstanding qualities as a teacher and as an evidence of the esteem of the Lambda Chapter of the Phi Beta Pi Medical Fraternity it presented, in 1944, to the St. Louis University School of Medicine, the "John Auer Lectureship" which enabled the school to bring to it distinguished scientists from various fields.

It is difficult to describe briefly Dr. Auer "the man" because of his versatility. He was devoted to his family and friends, an enthusiastic gardener, an ardent reader of the classics, spending much time with the French, German, and Latin authors, a critic in literature, art, and music, and a painter whose Saturdays always included a sketching trip which ended with a visit to his old friends in frames on the walls of the Art Museum. In addition to his deep love for the truth, he possessed an intense sympathy for mankind, and an ardent desire to aid in the creation of a better world. His leadership and fellowship will be missed by men in all walks of life.

GEORGE B. ROTH .

A COMPARISON OF THE EFFECTIVENESS OF DIHYDROERGOTAMINE (DHE-45) AND DIHYDROERGOCORNINE (DHO-180) IN THE PREVENTION OF CARDIAC IRREGULARITIES DURING CYCLOPROPANE ANESTHESIA¹

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In a pharmacological evaluation of dihydroergotamine methanesulfonate (DHE-45) reported by Orth and Ritchie (1), it was found that 0.4 mgm. of the drug per kgm. completely inhibited cardiac irregularities produced by the injection of a standard dose of epinephrine into dogs anesthetized with cyclopropane. Similar protective activity with an equal dosage of dihydroergocornine (DHO-180) was reported by Orth, Capps and Suckle (2). The purpose of the present study is a comparison of the relative effectiveness of these two drugs in the prevention of cyclopropane-epinephrine induced arrhythmias and also a preliminary note regarding trials and comparisons of DHO-180 and DHE-45 to prevent spontaneous cardiac irregularities arising clinically during cyclopropane anesthesia.

METHODS. Ten dogs and 4 monkeys (*Macacus rhesus*) were used in this comparative study. The animals were anesthetized with cyclopropane and oxygen mixtures and maintained on approximately 30 per cent cyclopropane in oxygen by an endotracheal to-and-fro absorption method. After equilibrating the animals on this mixture for 20 to 30 minutes, epinephrine was administered by a standard technic (3).

During the injection period electrocardiograms were recorded every 10 seconds. In the second minute tracings were made every 15 seconds and then records were made at intervals for a period of 5 minutes from the beginning of the epinephrine injection. The duration of ventricular tachycardia thus could be established for any given animal. The possibility of an adrenolytic action of cyclopropane (4) also was determined for each animal.

At subsequent periods with intervals of at least 3 days, the animals were anesthetized as above and given DHO or DHE, the drugs being administered separately to each animal on different days. Doses of 0.2 mgm. of the drugs per kgm. were diluted to a total volume of 5 cc. with normal saline and injected intravenously at the rate of 1 cc. each 10 seconds. Electrocardiograms were taken at appropriate intervals during these injections. After allowing 5 minutes to elapse following administration of the DH compound, the standard dosage of epinephrine previously found to be effective in eliciting ventricular tachycardia was given. If 0.2 mgm./kgm. of DH compound failed to protect against cardiac irregularities, some of the animals were given a subsequent 0.2 mgm./kgm. within 10 minutes of the first dose for the purpose of more accurate comparison of the two drugs. At 10 to 20 minute intervals after these injections, the standard dosage of epinephrine was repeated until cardiac irregularities again occurred or until 2 hours had elapsed after administration of the DH compound. Thus the amount and duration of protection could be determined for comparison of the drugs.

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² Resident in Anesthesiology from Helsingborg, Sweden.

In the *Macacus rhesus* when spontaneous irregularities occurred and normal rhythm could not be restored by inflation of the lungs with oxygen and decrease in depth of anesthesia, one of the DH compounds was injected intravenously. In all instances the irregularities occurring spontaneously were eliminated within 1 minute from the start of the injection. Thus it was possible to give epinephrine injections subsequently and determine the duration of protection.

In the preliminary clinical use of DHE and DHO for the protection of cardiac irregularities, 9 control and 23 test patients have been observed under a variety of operative conditions. The usual premedication of atropine or scopolamine alone or in combination with small doses of morphine sulfate was used. The three standard electrocardiographic tracings were taken, after which the DH compound was injected intravenously or intramuscularly. Initial dosage was 1 mgm. total amount. For succeeding patients this was increased to 5.5 mgm. of DHE or to 4 mgm. of DHO. Only 1 mgm. doses have been employed for intramuscular use. One to 3 minutes were used for the intravenous injections, during which time electrocardiographic records were taken. Two to 3 minutes following the injections, records were obtained again for the three standard leads. The induction of anesthesia with a cyclopropane-oxygen mixture was started within 5 minutes after completion of DH compound injection. Maintenance of anesthesia was with cyclopropane and oxygen using the to-and-fro absorption technique either with or without tracheal intubation.

The electrocardiographic beam, usually lead II, was observed continuously and tracings were made whenever cardiac irregularities were anticipated or seen. The usual graphic anesthesia record of pulse, blood pressure, respiratory rate and depth of anesthesia, along with other notations for correlation with the stage of the operation, was kept by the anesthetist.

RESULTS. In tables 1 and 2 are summarized the laboratory data. It can be noted from them that DHO unquestionably is a better protective agent in the 4 monkeys studied and that it appears to have some advantage over DHE in dogs. The adrenolytic effect of cyclopropane did not occur within the times noted in table 2 for duration of protection.

The clinical results up to the present time are summarized in table 3. Not only were arrhythmias more varied and frequent subsequent to the use of DHE than after the use of DHO, but those which arose were of greater possible danger. Three of the patients given DHE developed periods of ventricular tachycardia and in two of these instances it occurred within 10 minutes of the administration of the drug.

Oxygenation appeared to be good at the time tachycardia occurred and a relative overdose of cyclopropane seemed to be the cause. One of the 13 patients given DHO showed one short burst of ventricular tachycardia which occurred 30 minutes after the injection of 3 mgm. of the drug and at a time when there was cyanosis due to respiratory depression.

Nausea in 2 patients and vomiting in 1 occurred during the intravenous injection of DHO. Nausea or vomiting did not occur with the intravenous administration of up to 5.5 mgm. of DHE. The dosages were injected during a period of 3 minutes.

With the use of DHO there was a striking absence of the elevation of blood pressure that frequently occurs during the induction of cyclopropane anesthesia and there was a tendency for the systolic and diastolic pressure to become lower and remain lower than usual during the maintenance of anesthesia. This was not true in the patients treated with DHE.

Six of the 13 individuals given DHO exhibited periods of bradycardia with rates of under 50 per minute during cyclopropane anesthesia. None of the patients given DHE had such a degree of bradycardia.

Two of the individuals given DHO had a moderate bradycardia for 6 to 12 hours post-operatively. Otherwise there were no unusual post-operative effects.

Intestinal activity was of interest. Intraperitoneal operations were performed on 2 of the patients given DHO and observations were possible within 15 minutes of the start of the anesthetic. In both instances there was a marked contraction

TABLE 1

The protective action of Dihydroergotamine and Dihydroergocornine on cyclopropane-epinephrine tachycardia in dogs and monkeys

ANIMAL NO.	EPINEPHRINE DOSE	DURATION OF VENTRICULAR TACHYCARDIA		
		Control epinephrine response	DHE-45 (0.2 mgm./kgm.) and epinephrine	DHO-180 (0.2 mgm./kgm.) and epinephrine
Dogs				
	<i>mgm./kgm.</i>	<i>seconds</i>	<i>seconds</i>	<i>seconds</i>
1	.005	10	0	0
2	.01	15	0	0
3	.01	50	0	0
4	.01	20	0	0
5	.01	70	0	0
6	.02	10	Burst	0
7	.01	10	0	0
8	.01	50	40	Burst
9	.01	20	20	0
10	.01	70	0	Bursts
Monkeys				
1	.001	20	Bursts*	0*
2	.001	20	0*	0*
3	.005	40	120	Bursts
4	.005	50	40	0

* A dosage of 0.01 mgm./kgm. of epinephrine was used in these tests.

of the gastro-intestinal tract. A cholecystectomy was done on one of the DHE treated patients. Only the gallbladder was adequately observed and it was found to be small and contracted.

DISCUSSION. The number of dogs studied in this series is not sufficient and the differences between the protective ability of the two drugs in a given animal are too small to be certain that one drug is better than the other. Admittedly there is some day-to-day variation in the response of the individual animal. On the other hand, because of the clear-cut difference in the amount and duration of protection between DHO and DHE in the 4 monkeys, it seems fair to state that DHO is the better drug for this species.

In the dog and monkey, DHO, as well as DHE, prevents cyclopropane-epinephrine induced cardiac irregularities without harmful side effects. These

TABLE 2

Effects of dihydroergocornine and dihydroergotamine on cardiac irregularities produced deliberately in the dog and in the monkey

ANIMAL NO.	DH-COMPOUND		PROTECTION TO CYCLO-PROPANE-EPINEPHRINE CARDIAC IRREGULARITIES*		REMARKS
	Drug injected	Dosage	Dosage of epinephrine	Duration of protection	

Monkeys					
		mgm./kgm.	mgm./kgm.	min.	
1	DHE	0.2	0.01	None	Better protection with DHO
	DHO	0.2	0.01	70	
2	DHE	0.2	0.01	51	Better protection with DHO
	DHO	0.2	0.015	94	
3	DHE	(0.2 + 0.2)	0.005	None	Better protection with DHO
	DHO	(0.2 + 0.2)	0.005	38	
4	DHE	0.2	0.005	None	Better protection with DHO
	DHO	0.2	0.005	49	

Dogs					
1	DHE	0.2	0.005	58	Questionable advantage to DHO
	DHO	0.2	0.005	75	
2	DHE	0.2	0.01	111	Questionable advantage to DHO
	DHO	0.2	0.01	120+	
3	DHE	(0.2 + 0.2)	0.01	None	No advantage to either drug
	DHO	(0.2 + 0.2)	0.01	None	
4	DHE	0.2	0.01	None	No advantage to either drug
	DHO	0.2	0.01	None	
5	DHE	0.2	0.01	122	Approx. same protection with both drugs
	DHO	0.2	0.01	124	
6	DHE	(0.2 + 0.2)	0.02	28	Better protection with DHO
	DHO	0.2	0.02	68+	
7	DHE	(0.2 + 0.2)	0.01	23	Better protection with DHO
	DHO	0.2	0.01	54	
8	DHE	(0.2 + 0.2)	0.01	None	No advantage to either drug
	DHO	(0.2 + 0.2)	0.01	None	
9	DHE	(0.2 + 0.2)	0.01	None	No advantage to either drug
	DHO	(0.2 + 0.2)	0.01	None	
10	DHE	0.2	0.01	60	Better protection with DHE
	DHO	0.2	0.01	None	

* Ventricular tachycardia, ventricular premature contractions, or bundle branch block.

drugs also will eliminate spontaneous irregularities which occur in monkeys during cyclopropane anesthesia.

The clinical results up to the present time are far from being conclusive. Optimum dosage, route of administration, duration of action, and side-effects of these drugs for use during cyclopropane anesthesia in man can only be determined after many more patients are studied.

If the DH compounds do prove to be of value in the treatment or prevention of spontaneous cardiac irregularities arising during cyclopropane anesthesia, the results in these 23 clinical cases indicate that DHO would be the better drug to use. Kurtz, Bennett and Shapiro (5) made electrocardiographic observations on 41 patients during anesthesia with cyclopropane. Four of these patients

TABLE 3

The effect in patients of the use of DHO-180 and DHE-45 to prevent spontaneous cardiac irregularities as observed and recorded electrocardiographically during cyclopropane anesthesia

NUMBER OF PATIENTS	TOTAL DOSAGE INJECTED	CARDIAC IRREGULARITIES OCCURRED	NO CARDIAC IRREGULARITIES OCCURRED
DHO-180			
	mgm.		
3	1.0	1	2
7	2.0	4	3
3	4.0	1	2
DHE-45			
2	1.0	1	1
3	2.0	2	1
1	3.0	1	0
1	5.0	1	0
3	5.5	2	1

developed ventricular tachycardia of relatively long duration. In the one instance that ventricular tachycardia occurred during the use of DHO in 13 patients, its duration was only a matter of a few seconds. Thus, the definite impression has been gained that DHO affords some protection in man.

Certain aspects of the sympathicolytic activity of DHE-45 and DHO-180 have been studied by Bluntschli and Goetz (6). They conclude that DHO is the first known ergot derivative to act in a purely sympathicolytic fashion in man and they suggest that its site of action is in the higher sympathetic centers, i.e., the medulla and/or the hypothalamus. In the study of the mechanism of cyclopropane sensitization (7), it was found that tachycardia does not appear after decerebration, the production of lesions in the pons, or bilateral sympathectomy. These findings suggest that DHO might be a suitable drug to control spontaneous cardiac irregularities occurring in man during cyclopropane anesthesia.

Up to the present time it appears unlikely that a standard dosage of DHO or DHE can be determined for clinical usage which will prevent cardiac irregularities without producing disturbing side effects. A more reasonable aim would be

to determine a dose that would prevent the serious arrhythmias that occasionally occur in a well managed anesthetic. Whether the effects on blood pressure, pulse rate, and intestinal motility prove to be a hazard or an aid to clinical anesthesia is purely speculative at this time.

CONCLUSIONS

1. Dihydroergocornine (DHO-180) appears to be a more effective drug than dihydroergotamine (DHE-45) for the prevention of cyclopropane-epinephrine induced cardiac irregularities in dogs and monkeys.
2. The compounds stop spontaneous cardiac arrhythmias occurring during cyclopropane anesthesia in the *Macacus rhesus*.
3. Preliminary clinical trials in 23 patients are described.

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REFERENCES

1. ORTH, O. S., AND RITCHIE, G.: THIS JOURNAL, 90: 166, 1947.
2. ORTH, O. S., CAPPS, R. T., AND SUCKLE, H. M.: Fed. Proc., 6: 361, 1947.
3. ALLEN, C. R., STUTZMAN, J. W., SLOCUM, H. C., AND ORTH, O. S.: Anesthesiol., 2: 503, 1941.
4. STUTZMAN, J. W., AND ALLEN, C. R.: Proc. Soc. Exper. Biol. and Med., 47: 218, 1941.
5. KURTZ, C. M., BENNETT, J. H., AND SHAPIRO, H. H.: J. A. M. A., 106: 434, 1936.
6. BLUNTSCHLI, H. J., AND GOETZ, R. H.: Am. Heart J., 35: 873, 1948.
7. ALLEN, C. R., STUTZMAN, J. W., AND MEEK, W. J.: Anesthesiol., 1: 158, 1940.

THE EFFECT OF TOPICALLY APPLIED ANTIHISTAMINIC DRUGS ON THE MAMMALIAN CAPILLARY BED¹

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INTRODUCTION. In a recent review of the pharmacology of the antihistaminic drugs, Loew (1) stated, "It will be important to determine the extent to which the action of antihistamine drugs is referable to a direct effect upon capillaries. . . ." This statement leads to the present investigation in which it was decided that topical application of these drugs without pre- or post-histamine medication might show the site of action of the antihistaminic compounds insofar as the capillary bed itself was concerned.

EXPERIMENTAL. The meso-appendix of Wistar strain rats was exteriorized and arranged for perfusion and microscopic observation according to the method of Chambers and Zweifach (2). The animals used weighed from 135-220 gm. (average 150 ± 10 gm.). The anesthetic, sodium pentobarbital 45 mgm./kgm. intraperitoneally, was given in a total volume of not more than 0.15 cc. to reduce any untoward effects such as those described by Zweifach *et al.* (3). Further, as required to maintain the same depth of anesthesia additional anesthetic was given, usually not more than 0.05 cc. of the pentobarbital solution. The solutions of the drugs in Locke-Ringer's were prepared prior to use and maintained at $37.5^\circ \pm 0.2^\circ\text{C}$. until applied to the preparation. The perfusion fluid, Locke-Ringer's containing 1 per cent gelatin, was allowed to drip on the membrane at a temperature of $37.5^\circ \pm 0.2^\circ\text{C}$. maintained by an electrically heated and thermostatically controlled water bath of 12 l. capacity. All animals were standardized as to vasoconstrictor response of the capillary bed by the topical application of 0.1 cc. of $1:2 \times 10^4$ solution of epinephrine and direct observation of the precapillary sphincters of a selected vessel. Although all the vascular elements in the field were observed, all subsequent evaluations were made upon this vessel in a given animal and those which were not responsive were discarded. The antihistaminics and control drugs were made up in molar solutions so that better comparison of their vasoconstrictor potency could be obtained. The periods of observation after applying the drugs and washing to remove the drug were 5 and 10 minutes, respectively. Epinephrine, 0.1 cc. $1:2 \times 10^4$, was applied before and after each dilution of the drug to check not only the normality of the membrane but also to determine accurately the type of response observed. Further, to be certain that the drugs were acting at the same site as histamine, this drug was topically applied to the membrane at a dose of 0.1 cc. of a 0.0001 molar solution, this being the dose necessary to increase the rate of flow in the vascular bed without causing any deleterious effects in the animals. In order to rule out the effect of acetylcholine, this drug was also tested, and it was necessary not only to wash the membrane but to apply Pavatrine 0.1 cc. of 0.01 molar solution to obtain again a response to epinephrine. Further, to be certain that the effect of the drug was not due to the solvent used, 0.1 cc. of water was applied to the membrane after the study of the responses of each animal was completed. Also, when the response observed was doubtful, it was checked by applying either diphenhydramine or pyranisamine 0.1 cc. of a 0.01 molar solution. A control group was also tested with benzazoline because this drug, which contains the imidazole group, was known to be a potent vasodilator.

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RESULTS. The effects observed with the various drugs tested are given in tables I to VI in which the drugs are classified as to chemical structure. Further, lateral histograms (see tables) are used to enable the potency to be rapidly estimated. In the dilutions which were effective, the degree of local vasoconstrictor activity of the drugs was equivalent to that produced by the similar local effects

TABLE I
Ethanolamine derivatives

COMMON NAME	CHEMICAL NAME	MOLECULAR WEIGHT BASE	MOLAR CONCENTRATION					REMARKS
			1 M	0.1 M	0.01 M	0.001 M	0.0001 M	
Benadryl Diphenhydramine	β -dimethylamino ethyl benzhydryl ether HCl	255.35	*5/5	5/5	5/5	4/5	1/5	Precapillary sphincters completely closed at 0.001 M
Linadryl A-446	β - morpholino - ethyl benzhydryl ether HCl	297.33	5/5	5/5	0/5	0/5	0/5	Precapillary sphincters closed at 0.1 M
Decapryn Doxylamine	Benzyl - pyridyl-methyl - ethoxy-dimethyl - amine succinate	270.36	5/5	5/5	1/5	0/5	0/5	Precapillary sphincters closed at 0.1 M
SC 1694	β -dimethylamino ethyl benzhydryl ether salt of 8-chlorotheophyllin	255.35	0/5	0/5	0/5	0/5	0/5	Vasodilator (See table VI)
Prep. 204	Benzhydryl-oxy-methyl imidazoline HCl	266.33	5/5	5/5	5/5	3/5	0/5	Precapillary sphincters closed at 0.001 M
Tastromin 929F Thymoxyethyl-diethylamine	2 - isopropyl - 5 methyl phenoxyethyl diethylamine HCl	249.38	5/5	5/5	5/5	3/5	0/5	Precapillary sphincters closed at 0.001 M. Capillary hemorrhage in one animal

* The ratios signify the number of active vasoconstrictor responses over the number of animals used.

of epinephrine. However, diphenhydramine, pyranisamine, phenindamine and phenergan caused a definite decrease in the blood flow throughout the entire field. This was observed as a blanching of the membrane, and indicates that these drugs at a dilution of 0.0001 M are more potent vasoconstrictors than epinephrine at $1:2 \times 10^6$. In general, the vasoconstrictor activity of these drugs agrees with both the experimental and clinical results thus far reported.

DISCUSSION. The vasodilatation produced by such drugs as SC 1898 and SC 1742 is readily explained by their similarity to acetylcholine which also had a like

TABLE II
Thenyl derivatives

COMMON NAME	CHEMICAL NAME	MOLECULAR WEIGHT BASE	MOLAR CONCENTRATION					REMARKS
			1 M	0.1 M	0.01 M	0.001 M	0.0001 M	
Diatrin	N-dimethyl-N' - phenyl-N' - (2-thienyl) methyl-ethylenediamine HCl	260.39	*5/5	5/5	1/5	0/5	0/5	Precapillary sphincters completely closed at 0.1 M
Tagathen Chlorothen citrate	N-dimethyl-N' - pyridyl-N' - 5-chlorothenyl - ethylenediamine citrate	293.81	5/5	5/5	2/5	0/5	0/5	Precapillary sphincters closed at 0.01 M. Drug caused capillary hemorrhage in 3 animals at 0.1 M
Chlorothen	N-dimethyl-N' - pyridyl-N' - 5-chlorothenyl - ethylenediamine HCl	293.81	5/5	4/5	3/5	0/5	0/5	Precapillary sphincters closed at 0.01 M. Drug caused capillary hemorrhage in 3 animals at 0.1 M
Bromothen	N-dimethyl-N' - pyridyl-N' - 5-bromothenyl - ethylenediamine HCl	337.27	4/5	4/5	1/5	0/5	0/5	Precapillary sphincters completely closed at 0.1 M
Thenylene Histadyl Methapyrilene	N-dimethyl-N' - pyridyl-N' - thenylethyl - enediamine HCl	259.36	5/5	5/5	2/5	0/5	0/5	Precapillary sphincters closed at 0.01 M. Drug caused capillary hemorrhage in one animal at 0.1 M

* The ratios signify the number of active vasoconstrictor responses over the number of animals used.

action upon the capillary bed. Furthermore, these three compounds as well as histamine decreased the stickiness of the white cells causing them to enter the streamline flow. Epinephrine and the other antihistaminics caused the white cells to adhere to the vessel walls.

The vasodilatation produced by SC 1694 was probably due to the 8-chlorotheophyllin being more potent than the diphenhydramine part of the molecule. In particular this drug has a more potent action upon the larger vessels in the

TABLE III
Ethylenediamine derivatives

COMMON NAME	CHEMICAL NAME	MOLECULAR WEIGHT BASE	MOLAR CONCENTRATION					REMARKS
			1 M	0.1 M	0.01 M	0.001 M	0.0001 M	
Neontergan† Pyranisamine	N' - p - methoxy - benzyl - N' - pyridyl N - dimethyl ethylene diamine maleate	285.40	5/5*	5/5	5/5	5/5	0/5	Precapillary sphincters completely closed at 0.001 M
Pyribenzamine Tripeleennamine	N' - benzyl - N' - pyridyl - N - dimethyl - ethylene diamine HCl	255.37	5/5	4/5	3/5	1/5	0/5	Precapillary sphincters completely closed at 0.01 M
Neohetramine Thonzylamine	N - p - methoxy - benzyl - N' - pyramidyl - N - dimethyl ethylene - diamine HCl	287.40	5/5	5/5	5/5	5/5	3/5	G.I. infection sensitizing the preparation to the drug
" "	" "		5/5	5/5	0/5	0/5	0/5	Precapillary sphincters closed completely at 0.1 M
1571 F	N' - phenyl - N' - ethyl - N - diethylethylene diamine HCl	220.35	5/5	5/5	5/5	5/5	0/5	Precapillary sphincters closed at 0.001 M but not as potent as Neoantergan
Antistine Phenazoline	N' - phenyl - N' - benzylamino - methyl - imidazole HCl	265.35	5/5	5/5	5/5	3/5	0/5	Precapillary sphincters closed at 0.001 M but not as potent as Neoantergan

* The ratios signify the number of active vasoconstrictor responses over the number of animals used.

† Lessens effect of epinephrine, histamine, and H₂O.

preparation. In regard to vasodilatation by direct action on the muscular coating of the blood vessels, theophyllin is the most potent of all the xanthine drugs.

The finding that acetylcholine in high dilution produced a permanent vasodilatation unless neutralized by Pavatrine was to be expected because of the predominant role of the cholinergic fibers in the nervous control of the intestinal tract.

The inactivity of benzazoline, which has been shown to be a potent vasodilator on the capillaries of the rabbit's ear (4) and hind limb preparations (5), may have been due to the animal used (the rat). However, Littner (6) reported that benzazoline had no peripheral vasodilator effect in the toad, dog, rabbit and rat. Further, Braun (7), after using the rat, concluded that the site of action of the drug was proximal to, but not on, the capillaries themselves. Regardless of the

TABLE IV
Thiodiphenylamine derivatives

COMMON NAME	CHEMICAL NAME	MOLECULAR WEIGHT BASE	MOLAR CONCENTRATION					REMARKS
			1 M	0.1 M	0.01 M	0.001 M	0.0001 M	
SC 1627 3015 RP	N-dimethylamino ethyl thiodiphenylamine HCl	270.26	5/5*	5/5	4/5	0/5	0/5	Precapillary sphincters closed at 0.01 M
Phenergan 3277 RP	N-dimethylamino - isopropyl thiodiphenyl - amine HCl	284.41	5/5	5/5	5/5	5/5	0/5	Precapillary sphincters completely closed at 0.001 M. Equal to Neo-antergan
SC 1923	N-methyl ethanol amino ethyl thiodiphenyl - amine HCl	300.25	5/5	5/5	5/5	0/5	0/5	Precapillary sphincters closed at 0.01 M
SC 1898	N-dimethyl ethanol amino ethyl thiodiphenylamine bromide	395.20	0/5	0/5	0/5	0/5	0/5	Vasodilator (See table VI)
SC 1742	N - trimethyl - amino ethyl thiodiphenyl - amine chloride	320.75	0/5	0/5	0/5	0/5	0/5	Vasodilator (See table VI)

* The ratios signify the number of active vasoconstrictor responses over the number of animals used.

exact site of action it is known that benzazoline definitely inhibits both injected epinephrine (8, 9) and sympathin released by adrenergic nerve stimulation (10-12). This is important because we observed no epinephrine inhibition or reversal after topical application of benzazoline to the rat meso-appendix capillary bed. Histamine, which acts directly upon smooth muscle, caused dilation and phenazoline and 204 both caused vasoconstriction. All four of these compounds contain the imidazole group and the latter two are analogues of Antergan and diphenhydramine, respectively. Thus, from a structural viewpoint it appears that the aromatic substituents at the opposite end of the aliphatic chain play a more important part than the imidazole group insofar as vasoconstrictor potency is con-

cerned. Furthermore, the results herein presented indicate that the vasoconstrictor activity of the antihistaminic drugs is directly on the muscle cells of the precapillary sphincters and is not mediated through the adrenergic nerves. This is in accord with the statement of Wells and Morris (13) that an antihistaminic drug competes with histamine for its site of action.

TABLE V
Miscellaneous compounds

COMMON NAME	CHEMICAL NAME	MOLECULAR WEIGHT BASE	MOLAR CONCENTRATION					REMARKS
			1 M	0.1 M	0.01 M	0.001 M	0.0001 M	
SC 887	Diethylamino - ethanol 9-10, dihydroan - thracene carboxylate HCl	323.28	5/5*	5/5	5/5	0/5	0/5	Precapillary sphincters closed at 0.01 M
Trimeton	1 - phenyl - 1 - (2-pyridyl)-3-di-methylamino propane	240.34	5/5	5/5	4/5	0/5	0/5	Precapillary sphincters closed at 0.01 M
Prophepyrid-amine								
Pavatrine			5/5	5/5	2/5	0/5	0/5	
Thephorin† Phenindamine	2 - methyl - 9 - phenyl 2,3,4,9-tetra - hydro - pyridinolene hydrogen tartrate	260.34	4/5†	5/5	5/5	5/5	0/5	Animal died.† Precapillary sphincters closed completely at 0.001 M, equal to Neoantergan
Trasentine Adiphenine	Diethylamino - ethyl ester of diphenylacetic acid HCl	311.23	0/5	0/5	0/5	0/5	0/5	No effect on capillary bed
Distilled water			145 145					Complete blanching of the membrane

* The ratios signify the number of active vasoconstrictor responses over the number of animals used.

† Lessens effect of epinephrine and histamine.

From the results herein presented, it appears that drugs having a molecular weight from 249 to 287 are more potent than those of higher molecular weight. Further, it appears that substitution of a thenyl or halogenated thenyl group for the benzyl or pyridyl group decreases the vasoconstrictor potency, although the molecular weight may be maintained within the optimal range. Also, it can be seen that cyclization of the terminal amine group to produce a morpholine group

decreases the activity while cyclization to form the imidazole group has little effect on the potency as measured on the capillary bed.

In the rabbit and dog, increased capillary permeability has been measured by following the rate of extravasation of dyes or India ink after the intradermal injection of histamine. Diphenhydramine (14-17), tripeleennamine (14, 18, 19) thonzylamine (16, 20, 21), pyranisamine (22-24), doxylamine (25) and Diatrin

TABLE VI
Vasodilator compounds

COMMON NAME	CHEMICAL NAME	MOLECULAR WEIGHT BASE	MOLAR CONCENTRATION					REMARKS
			1 M	0.1 M	0.01 M	0.001 M	0.0001 M	
Acetylcholine chloride	—	181.59	5/5*	5/5	5/5	5/5	5/5	Complete relaxation of capillary bed, no recovery on washing
SC 1742	N - trimethyl - amino ethyl thiodiphenyl amine chloride	320.75	5/5	5/5	5/5	5/5	5/5	Same but recovered on washing. Also see table IV. 3 animals did not respond to histamine or distilled water
SC 1694	β -dimethylamino-ethyl benzhydryl ether salt of 8-chlorotheophyllin	255.35	5/5	5/5	5/5	0/5	0/5	No effect on capillaries. Relaxation of met-arterioles and arterioles. See table I
Priscol	2-benzyl imidazoline HCl	160.21	0/5	0/5	0/5	0/5	0/5	No effect on capillary bed
Histamine	β - imidoazoly - 4 ethylamine HCl	111.09	0	0	0	0	$\frac{145}{145}$	Relaxation of vessels and increased blood flow through entire area

* The ratios signify the number of active vasoconstrictor responses over the number of animals used.

(26) are all able to diminish or prevent the local accumulation of dye which occurs after the intradermal injection of histamine. The other antihistaminics would be expected to have a similar action differing only in the doses required to prevent the extravasation of the dye. The wheal and flare reaction that occurs after the intradermal injection of histamine in man is also diminished or prevented by premedication with the antihistaminic drugs (27). The reduction of the tuberculin skin reaction after antihistaminic medication has also been reported (28, 29), as

has the reduction in the egg white edema in the rat (30, 31). Further, Halpern (32) found that capillary resistance was elevated in allergic, but not in normal, subjects after medication with phenergan, Antergan or pyranisamine. Phenergan was the most potent in this respect. The action of histamine in these cases is purely a local one and the doses of antihistaminics employed are within the range herein reported as causing vasoconstriction on the capillary bed. Further, it is logical to assume that whether histamine itself acts to relax the precapillary sphincters or has a general relaxant effect upon the other vessels of the peripheral vascular system (metarterioles and arterioles), the overall effect would be one of congestion ending in the loss of fluid into the interstitial spaces. Any mechanism which prevents this relaxation and congestion would tend to decrease capillary permeability. The vasoconstrictor action of the antihistaminic drugs on the precapillary sphincters reduces the flow through the capillary bed and thus would reduce capillary permeability and the extravasation of dyes, India ink or plasma into the surrounding tissues. Further, the observation that when histamine was applied to the capillary bed the cell wall and/or the leucocytes tended to be less sticky (enter the streamline flow of the erythrocytes) might lead one to assume that the leucocytes play a mechanical part in either decreasing or increasing capillary permeability. Both the antihistaminics and epinephrine caused an opposite effect, namely, that of increasing the stickiness of the cell wall and/or the white cells causing those cells to remain more firmly attached to the cell walls of the vessels. Furthermore, by acting as vasoconstrictors at the precapillary sphincters, the antihistaminics would prevent histamine from acting without decreasing the total amount of circulating histamine. Pellerat and Murat (33) have shown that after antihistaminic therapy the circulating histamine was increased 6 to 12 fold for more than 24 hours. Staub (34) showed that intravenous epinephrine increased blood histamine levels and prior medication with intravenous phenazoline diminished this rise. Thus blocking the action did not result in too great a decrease in the histamine blood level. Furthermore, after the antihistaminic effect has worn off, there is a tendency for histamine to again produce its vascular effects.

This discussion of the capillary action of histamine does not take into consideration the other vascular effects of the drug. However, it has been shown that vasodepressor action (20, 21, 25, 32, 35-51) and the vasoconstrictor action on the larger arteries (52) are all blocked by the antihistaminic drugs. Such action would support the belief that both histamine and the antihistaminics act at the same site and that there is a different muscular area concerned with each action.

SUMMARY

1. It has been shown that the antihistaminic drugs, not related to acetylcholine, have a vasoconstrictor action on the precapillary sphincters of the mammalian capillary bed, while histamine has a vasodilator action. This demonstrates that both drugs compete for the same site of action.

2. It has been shown that acetylcholine and antihistaminic compounds having the choline group exert a vasodilator effect upon the capillary bed. Further, al-

though washing the bed counteracts the effect of the latter compounds, it is necessary to apply an antispasmodic drug to counteract the effect of the former.

3. It has been shown that antispasmodic drugs have at best only a slight vasoconstrictor action upon the capillary bed.

4. The vasodilator action of 8-chlorotheophyllin is more potent than the vasoconstrictor action of diphenhydramine. However, the effect is not upon the pre-capillary sphincters but upon the vessels having more muscular coats (arterioles).

5. The vasoconstrictor effect produced by the antihistaminics on the capillary bed is produced at dilutions which are of the same order of magnitude as those obtained in animals or humans after oral or intravenous administration. Further, such vasoconstriction explains the decrease in Trypan Blue extravasation in animals, the decrease in the wheal and flare reaction in humans and the reduction in the tuberculin skin reaction.

6. The cyclization of the terminal amine group to form a morpholine group decreases the vasoconstrictor action of diphenhydramine. However, cyclization to form the imidazole group does not decrease the vasoconstrictor action of an antihistaminic. The substitution of a thenyl or halogenated thenyl group for either the phenyl or pyridyl group results in a decrease in vasoconstrictor action.

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REFERENCES

1. LOEW, E. R.: *Physiol. Rev.*, **27**: 542, 1947.
2. CHAMBERS, R., AND ZWEIFACH, B. W.: *Am. J. Anat.*, **75**: 173, 1944.
3. ZWEIFACH, B. W., HERSHEY, S. G., ROVENSTEIN, E. A., AND CHAMBERS, R.: *Proc. Soc. Exper. Biol. & Med.*, **56**: 73, 1944.
4. MEIER, R., AND MUELLER, R.: *Schweiz. Med. Wschr.*, **69**: 1271, 1939.
5. MEIER, R., AND MEYER, R. T.: *Schweiz. Med. Wschr.*, **71**: 1206, 1941.
6. LITTNER, M.: *Sem. Med. (Buenos Aires)*, **60**: 794, 1944.
7. BRAUN, H.: *Derm. Wschr.*, **115**: 1003, 1942.
8. AHLQUIST, R. P., AND WOODBURY, R. A.: *Fed. Proc.*, **5**: 161, 1946.
9. HERMAN, H., AND VIAL, H.: *Compt. rend. soc. de biol.*, **136**: 803, 1942.
10. HERMAN, H., JOURDON, F., AND BONNET, V.: *Compt. rend. soc. de biol.*, **135**: 1653, 1941.
11. CHESSE, D., AND YONKMAN, F. F.: *Fed. Proc.*, **4**: 114, 1945.
12. CHESSE, D., AND YONKMAN, F. F.: *Proc. Soc. Exper. Biol. & Med.*, **61**: 127, 1946.
13. WELLS, J. A., AND MORRIS, H. C.: *Fed. Proc.*, **4**: 140, 1945.
14. DREISBACH, R. H.: *Fed. Proc.*, **6**: 323, 1947; *J. Allergy*, **18**: 397, 1947.
15. LAST, M. R., AND LOEW, E. R.: *THIS JOURNAL*, **89**: 81, 1947.
16. BUKANTZ, S. C., AND DAMMIN, G. J.: *Science*, **107**: 224, 1948.
17. TRAUB, F. B., FRIEDMAN, U., AND LANDSTADT, D.: *J. Allergy*, **18**: 273, 1947.
18. RENNICK, B., CHESSE, D., HAYS, H. W., MATHIESON, D., MAYER, R. L., AND YONKMAN, F. F.: *Fed. Proc.*, **4**: 133, 1945.

19. MAYER, R. L., AND KULL, F. C.: *Proc. Soc. Exper. Biol. & Med.*, **66**: 392, 1947.
20. SCUDI, J. V., REINHARD, J. F., AND DREYER, N. B.: *J. Allergy*, **19**: 184, 1948.
21. REINHARD, J. F., AND SCUDI, J. V.: *Proc. Soc. Exper. Biol. & Med.*, **66**: 512, 1947.
22. CULLUMBINE, H.: *Nature*, **159**: 841, 1947.
23. BONVALLET, M., AND DECOURT, P.: *Compt. rend. soc. de biol.*, **138**: 224, 1944; *ibid*, **138**: 305, 1944.
24. HUNTER, R. B.: *Lancet*, **252**: 672, 1947.
25. BROWN, B. B., AND WERNER, H. W.: *J. Lab. Clin. Med.*, **33**: 325, 1948.
26. ERCOLI, N., SCHLACHTER, R. J., HUEPER, W. C., AND LEWIS, M. N.: *THIS JOURNAL*, **93**: 210, 1948.
27. HALEY, T. J.: *J. Amer. Pharm. Assoc.*, **37**: 383, 1948.
28. BRETON, A.: *Compt. rend. soc. de biol.*, **137**: 254, 1943.
29. SARBER, R. W.: *Am. Rev. Tuberculosis*, **57**: 504, 1948.
30. BROWN, B. B., AND WERNER, H. W.: *Ann. Allergy*, **6**: 122, 1948.
31. LEGER, J., AND MASSON, G.: *Am. J. Med. Sci.*, **214**: 305, 1947.
32. HALPERN, B. N.: *Acta Allergologica*, **1**: 3, 1948.
33. PELLERAT, J., AND MURAT, M.: *Compt. rend. soc. de biol.*, **140**: 297, 1946.
34. STAUB, H.: *Helv. Physiol. Pharmacol. Acta*, **4**: C54, 1946.
35. PAGE, I. H., AND GREEN, A. A.: *Fed. Proc.*, **5**: 78, 1946.
36. BOVET, D., HORCLOIS, R., AND FOURNEL, J.: *Compt. rend. soc. de biol.*, **138**: 165, 1944.
37. HALPERN, B. N.: *Arch. internat. Pharmacodyn. et de Therap.*, **68**: 339, 1942.
38. MARSH, D. F., AND DAVIS, A. J., JR.: *THIS JOURNAL*, **89**: 234, 1947.
39. DREYER, N. B., AND HARWOOD, D.: *Proc. Soc. Exper. Biol. & Med.*, **66**: 515, 1947.
40. SHERROD, T. R., LOEW, E. R., AND SCHLOEMER, H. F.: *THIS JOURNAL*, **89**: 247, 1946.
41. LEHMANN, G., HAGEN, E., BARBAROW, G., AND ROE, M.: *Fed. Proc.*, **6**: 350, 1947.
42. DEWS, P. B., AND GRAHAM, J. D. T.: *Brit. J. Pharmacol.*, **1**: 278, 1946.
43. YONKMAN, F. F., OPPENHEIMER, E., RENNICK, B., AND PELLET, E.: *THIS JOURNAL*, **89**: 31, 1947.
44. WELLS, J. A., MORRIS, H. C., BULL, H. B., AND DRAGSTEDT, C. A.: *THIS JOURNAL*, **85**: 122, 1945.
45. BOQUET, A.: *Presse Med.*, **51**: 93, 1943.
46. RAMANAMANJARY, W.: *Compt. rend. soc. de biol.*, **138**: 480, 1944.
47. HALPERN, B. N.: *Bull. soc. chim. biol.*, **29**: 309, 1947.
48. WELLS, J. A., MORRIS, H. C., AND DRAGSTEDT, C. A.: *Proc. Soc. Exper. Biol. & Med.*, **61**: 104, 1946.
49. YONKMAN, F. F., HAYS, H. W., AND RENNICK, B.: *Fed. Proc.*, **4**: 144, 1945.
50. CLIMENKO, D. R., HOMBURGER, E., AND MESSER, F. H.: *J. Lab. Clin. Med.*, **27**: 289, 1941.
51. MINARD, D., AND ROSENTHAL, S. R.: *Proc. Soc. Exper. Biol. & Med.*, **44**: 237, 1940.
52. CAPRARO, V.: *Farm. sci. e tec. (Pavia)*, **2**: 98, 1947.

STUDIES OF THE ACTIONS OF 4-AMINO-PTEROYLGLUTAMIC ACID IN RATS AND MICE¹

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The cardinal signs of pteroylglutamic acid (PGA, folic acid) deficiency in mammals are disorders of hematopoiesis and of the digestive tract. Manifestations of severe deficiency include macrocytic anemia and granulocytopenia with hypoplasia of bone marrow involving erythro- and myelopoiesis. Megaloblastosis is found in monkeys and man. The lesions of the digestive tract include necrosis of the buccal and intestinal mucosa. Diarrhea is a feature. Current knowledge of the role of the vitamin in mammalian nutrition is exhaustively treated in a recent review by Jukes and Stokstad (1).

Attempts to develop structural analogs which antagonize the actions of PGA have been successful. Impure preparations of relatively low potency, presumably containing x-methyl analogues of PGA, were the first antagonists to accelerate and enhance the vitamin-deficiency when fed with PGA-deficient diets (2, 3, 4). As expected of competitive antagonists (5), the effects of these preparations were readily reversed by PGA. More recently a congener in which the 4-hydroxy group of PGA was replaced by an amino substitution (6) (4-amino-pteroylglutamic acid) was found to exert a fulminating toxic effect in laboratory animals. The lesions produced by this compound were difficult to prevent by simultaneous administration of PGA. Reports have appeared concerning its toxicity in several laboratory species (7, 8, 9) and further interest in the compound followed the announcement of its inhibitory actions against Rous sarcoma in young chicks (10) and acute leukemia in children (11). It appeared, therefore, of importance to explore the sites of its action and to assess whether the lesions produced by the agent could be considered to result from an antagonism of folic acid. For these reasons the study here reported was initiated in mice and rats. A subsequent report will deal with its actions in dogs (12).

PROCEDURE. Albino mice (AKM strain) and rats (Wistar strain) of both sexes served as experimental subjects. The animals had been raised and were maintained during the investigations on standard laboratory diets. With exceptions, mentioned below, the weights of mice were between 17.5 and 22.5 grams and of rats, between 100 and 200 grams.

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Solutions of 4-amino-PGA³ were prepared fresh daily for immediate use in 0.9 per cent NaCl. They were made from ampules containing measured samples which had been neutralized before drying, or by the addition of 2 molar equivalents of NaHCO₃ to weighed amounts of the free acid. Doses were administered in the constant volume of 0.5 cc. per 20 gram mouse and 1.0 cc. per 100 gram rat. Standard procedures, as recommended by Wintrobe (13), were used to investigate the blood of rats. Samples of 2 or more cc. of blood were obtained in heparinized syringes at time of sacrifice from deep cuts across the axillary vessels of stunned rats. Bone marrow samples were obtained from long bones. Femoral shafts were connected to small syringes by means of rubber tubing and their contents expelled by air-pressure. Marrow smears were stained by the Jenner-Giemsa technic. When possible blocks of marrow were fixed in Vandergrift's reagent and, after embedding, stained with hematoxylin-phloxin-eosin and Giemsa. Representative samples of spleen, liver, duodenum, jejunum, ileum, colon, mesenteric lymph node, sternum, and thymus were treated likewise.

RESULTS. Toxicity. The LD₅₀ of 4-amino-PGA in animals receiving single doses, as estimated graphically (14) from the data of table 1, was 1.9 ± 0.3 mgm. per kgm. in mice and 4.5 ± 1.4 mgm. per kgm. in rats. Several interesting features of the toxic actions may be noted in table 1. Although the agent was administered parenterally in doses which exceeded the LD₅₀ at least tenfold, no animal succumbed earlier than the third day. Oral administration in rats was as effective as parenteral injection. Furthermore, 4-amino-PGA, when given daily in fractions of the lethal dose, appeared to be almost as toxic in mice and possibly more toxic in rats than when given in single doses.

Antagonism of 4-amino-PGA. Attempts to reduce toxicity by treatment of animals with PGA met with limited success. Since the daily dosage of PGA employed was near the maximum tolerated by mice, it was not possible to explore the antagonism of the agents beyond the limits shown in table 2. Within this range the LD₅₀ of 4-amino-PGA was raised several fold by repeated administration of the vitamin. A similar result was obtained with large doses of pteroyltriglutamic acid (PTGA). It is interesting to note that the course of fatal intoxication was not appreciably altered in those animals which succumbed in spite of treatment with either PGA or PTGA. Most succumbed during the third or fourth day following 4-amino-PGA administration.

In view of possible interrelationships among the actions of PGA, the anti-pernicious-anemia factor in liver, and thymine (1) a few observations were made to assess the effects of the two last mentioned substances on the toxicity of 4-amino-PGA. When used as described for PGA and PTGA in table 2, refined liver extract and thymine in daily doses of 10 units per kgm. and 500 mgm. per kgm., respectively, failed to alter the effects of both 12.5 and 3.1 mgm. per kgm. of the toxic agent.

Course of Intoxication. During the first 24 hours following the administration of fatal doses of 4-amino-PGA the behavior and appearance of mice and rats were unaltered. Moderate losses in weight were noted by the end of the first day. Subsequently debilitation set in and progressed steadily to the extent that in-

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toxicated animals lost approximately 20 per cent of their initial weight within 72 hours. Severe, watery diarrhea appeared 48 hours after poisoning. The feces

TABLE 1
Toxicity of 4-amino-PGA in mice and rats

SPECIES	ROUTE OF ADMINISTRATION	NO. OF INJECTIONS	DOSAGE		MORTALITY	DAY OF DEATH		
			Single	Total		3 to 4	5 to 7	8 to 14
Mouse	Intraperitoneal	1	100	100	6/6	6	—	—
		1	25	25	6/6	6	—	—
		1	12.5	12.5	34/34	30	3	1
		1	6.3	6.3	11/12	10	1	—
		1	3.1	3.1	26/40	22	4	—
		1	1.6	1.6	9/18	8	1	—
		1	0.8	0.8	2/18	1	1	—
		1	0.4	0.4	3/6	—	3	—
		1	0.2	0.2	3/6	1	1	1
		5	1.6	8.0	6/6	2	4	—
		7	0.8	5.6	12/12	2	9	1
		7	0.4	2.8	7/12	—	5	2
		7	0.2	1.4	2/6	—	1	1
		7	0.1	0.7	0/3	—	—	—
		7	0.05	0.35	0/3	—	—	—
Rat	Intraperitoneal	1	40	40	6/6	3	3	—
		1	20	20	5/6	2	3	—
		1	10	10	5/6	2	3	—
		1	5	5	4/6	1	2	1
		1	2.5	2.5	2/6	—	1	1
		1	1.25	1.25	0/6	—	—	—
		4	1.0	4.0	4/4	3	1	—
		4	0.5	2.0	4/4	2	2	—
		8	0.25	2.0	6/10*	—	6	—
		8	0.125	1.0	0/6†	—	—	—
	Oral	1	40	40	5/6	3	2	—
		1	20	20	5/6	2	2	1
		1	10	10	4/6	3	1	—
		1	5	5	2/6	1	—	1
		1	2.5	2.5	2/6	2	—	—
		1	1.25	1.25	0/6	—	—	—

* Two surviving animals sacrificed on seventh day for hematologic and pathologic study; one of pair exhibited severe debilitation and diarrhea.

† All sacrificed on tenth day for hematologic and pathologic study; at this time the weights in percent of initial were as follows: 89, 101, 104, 104, 110, 133.

were yellowish-brown in color and, terminally, grossly stained with blood. The diarrhea persisted as a prominent feature of fatal intoxication in both mice and rats and contributed to their dehydrated and depressed appearance at death.

Animals surviving minimum lethal doses exhibited transient retardation in growth or actual loss of about 5 per cent of body weight during the first week. (Untreated animals of similar age from the same colony gain consistently 1 to 2 per cent of body weight per day.) Their subsequent recovery was rapid.

Pathology. The lesions caused by single, fatal doses of 4-amino-PGA were studied in rats receiving 40 mgm. per kgm. intraperitoneally. The animals were sacrificed at various intervals as noted in table 3 ("acute" group).

Effects of chronic administration of the agent were observed in a limited group of animals receiving either 0.25 or 0.125 mgm. per kgm. per day (PI and PII, table 3) and in a larger series of 15 males and 14 females which received the doses shown in figure 1 and table 3 (groups I to V). Animals of the latter series were sacrificed when they failed to gain or lost weight for at least 4 consecutive days.

TABLE 2
Effect of single doses of 4-amino-PGA in mice treated with PGA and PTGA

TREATED WITH	DOSE OF 4-AMINO-PGA mgm./kgm.	MORTALITY
PGA*	12.5	15/18
	3.1	2/18
	1.6	0/6
PTGA†	12.5	4/6
	6.3	1/6
	3.1	3/12
	1.6	0/6

* 47 mgm./kgm. administered intraperitoneally once daily at -2, -1, 0, 1, and 2 days.

† 500 mgm./kgm., once daily, as for PGA.

Only 4 animals of this series, all females, succumbed before sacrifice. The remaining females exhibited signs of poisoning before the end of the first 7 weeks and were included among the first 4 groups of animals (groups I to IV, table 3). Group V consisted of 8 male rats. Their gain in body weight was not seriously impaired until the initial dose had been increased fourfold.

1) *Lesions of blood and hematopoietic tissue.* The femoral marrow of rats receiving 40 mgm. per kgm. was converted from a greyish-red, gelatinous staff of material to a darker, more fluid substance by the twelfth hour after treatment. Progressive liquefaction of the marrow occurred until at 72 hours only purple fluid could be expelled from the femur. In the peripheral blood marked granulocytopenia and reticulocytopenia and a moderate lymphopenia developed simultaneously. In table 3 are shown changes in peripheral blood and bone marrow. It is evident that within 48 hours erythropoiesis and myelopoiesis were severely inhibited. The elevated values for lymphoid cells in the femoral contents taken from 48- and 72-hour animals were due to replacement of marrow tissues by blood. Hemoconcentration became evident at 48 hours when diarrhea first appeared.

Ill-defined areas of degeneration were observed in sternal and femoral marrow as early as 6 and 12 hours after the administration of 40 mgm. per kgm. of 4-amino-PGA. After 24 and 48 hours the sinus and capillary network of the marrow became prominent due to depletion in erythro- and myelopoiesis. Between 48 and 72 hours the hematopoietic tissues largely vanished from the marrow leaving a network of capillaries and sinusoids filled with blood. Capillary endothelial lining and histiocytes were prominent in smears and sections. The remainder of the original marrow consisted for the most part of eosinophils, mega-

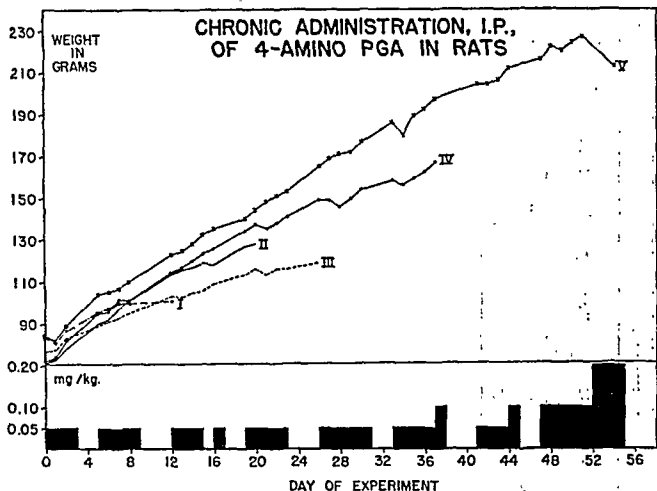


FIG. 1. GROWTH IN BODY-WEIGHT OF RATS RECEIVING REPEATED, INTRAPERITONEAL INJECTIONS OF 4-AMINO-PGA. DAILY DOSAGE INDICATED BY SOLID COLUMNS ALONG ABSCISSA

karyocytes, and a few basophil normoblasts and erythroblasts. Pyknosis was common in these cells. In the marrow smears lymphocytes were prominent due to admixture of peripheral blood since no lymph follicles or aggregations of these elements were found in sections.

In animals receiving 0.25 or 0.125 mgm. per kgm. of 4-amino-PGA daily for periods of 5 to 9 days (groups PI and PII, table 3) the hematological changes resembled those described above in the "acute" group 48 and 72 hours after poisoning. However, changes in hematopoietic tissues developed less rapidly and were less severe when small daily doses of 0.05 mgm. per kgm. were given (groups I to V, figure 1 and table 3). No significant changes were found in animals of group I and only one of group II and one of group III evidenced marrow depletion. Moderate depletion was found in the 3 animals of group IV.

TABLE 3
Hematological data from rats treated with 4-amino-PGA
(Number of animals used indicated in brackets)

GROUP	DOSAGE $\frac{\text{mgm.}}{\text{kgm./day}}$	NUMBER OF INJECTIONS	DAY OF SACRIFICE	PERIPHERAL BLOOD							FEMORAL MARROW								
				W.B.C. $\frac{1 \text{ c.mm.}}{\times 10^{-4}}$	PMN. %	MNC. %	Retic. %	Herit. $\frac{\text{cc.}}{100 \text{ cc.}}$	M.C.V. μ^3	M.C.H.C. $\frac{\text{g.}}{100 \text{ cc.}}$	Condition	PMN-N. %	PMN-EOS %	N Myel. %	Myelc. %	Mbl. %	Lymph. %	Normbl. %	
Control	40	1	5	Mean . . .	6.9 (11)	82 (11)	11 (6)	38.4 (6)	56 (6)	34 (6)	S (11)	33 (12)	6 (12)	9 (12)	8 (12)	2 (12)	9 (12)	35 (12)	
				Range . . .	2.7-13.9	12-29	7-17	37.4-41.4	52-62	31-38		10-57	2-20	4-13	3-16	1-5	3-21	10-70	
Acute	40	1	5	Mean . . .	20.4	15	5	40.0	33	33	F	79	7	5	0	0	1	7	
				Range . . .	5.5-55	52-62	5	31.8	31	F	22	4	49	0	0	4	2	17	
		1		Mean . . .	8.9	5	5	44.6	36	36	SF	27	4	5	2	1	4	17	
				Range . . .	7.7-10	90-90	24	38.4	59	59	SF	28	2	25	4	2	11	45	6
		2		Mean . . .	5.3	28	72	32.7	71	32	F	40	20	25	7	1	20	8	
				Range . . .	2.1-16	84-84	10	38.5	58	31	F	34	12	10	9	1	20	8	4
				Mean . . .	16.8	5	2	51.8	56	31	F	45	6	6	5	5	32	36	
				Range . . .	8.7-26	6-8	8	40.0	56	31	F	28	4	10	5	5	32	36	4
		2		Mean . . .	2.0	3	6	52.8	34	34	F	2	7	3	0	0	84	4	
				Range . . .	8.5-50	50-50	2	52.5	34	34	F	34	8	8	5	1	40	4	4
		3		Mean . . .	1.1	3	1	52.4	48	20	F	3	9	0	2	0	82	4	
				Range . . .	2.4-98	2-98	1	52.4	48	20	F	3	9	0	2	0	82	4	0
P I	0.25	5	7	Mean . . .	1.0 (6)	1 (8)	99 (8)	49.3 (2)	58 (1)	34 (2)	F (6)	3 (8)	6 (8)	2 (8)	2 (8)	0 (8)	82 (8)	2 (8)	
				Range . . .	1.0-2.5	0-4	90-100	50.9-61.6	33-34	33-34		0-13	3-10	0-5	0-7	0-1	67-92	0-3	0-3
P II	0.125	8	10	Mean . . .	6.3	14	86				S	55	4	3	22	4	7	3	
				Range . . .	2.2	6	94				F	6	15	2	2	0	67	6	6
I	0.05	7-12	12-20	Mean . . .	3.4 (6)	9 (6)	91 (6)	38.3 (3)			F (6)	13 (6)	13 (6)	11 (6)	12 (6)	4 (6)	39 (6)	8 (6)	
				Range . . .	1.3-5.2	1-36	65-99	30.3-50.0	23-39	23-39		2-32	5-22	6-23	4-18	1-8	29-61	3-15	3-15
II	0.05	14	22	Mean . . .	8.5 (4)	51 (5)	42 (5)	15 (3)	38.3 (3)	21 (3)	S (4)	17 (4)	5 (4)	24 (4)	12 (4)	0.5 (4)	0.5 (4)	41 (4)	
				Range . . .	0.5-11.8	23-67	25-75	8-22	30.3-50.0	23-39		9-23	0-18	18-36	6-19	5-10	0-2	22-65	11 (4)
III	0.05	15-17	20-28	Mean . . .	5.4 (4)	24 (4)	76 (4)	8 (4)	35.9 (4)	34 (4)	S (3), F (1)	25 (4)	7 (4)	14 (4)	11.5 (4)	7.5 (4)	5 (4)	29 (4)	
				Range . . .	2.2-9.9	6-56	44-94	2-15	32-43.5	31-38		16-33	2-13	8-17	7-16	3-10	1-16	18-34	29 (4)
IV	0.05-0.10	30	48	Mean . . .	11.3 (5)	23 (5)	75 (5)	10 (5)	43.0 (5)	60 (5)	S (4), SF (1)	37 (5)	4.5 (5)	14 (5)	7 (5)	0 (5)	2 (5)	35 (5)	
				Range . . .	4.4-18.3	6-56	60-91	4-17	35.0-50.5	52-71		16-33	2-8	8-22	3-11	1-3	27-41	18-34	35 (5)
V	0.05-0.20	30 and 37	54 and 65	Mean . . .	5.9 (3)	16 (3)	84 (3)	7 (3)	40.0 (3)	33 (3)	S (3)	41 (3)	19 (3)	11 (3)	3 (3)	3 (3)	4 (3)	18 (3)	
				Range . . .	3.5-8.2	0-30	70-94	4-10	37.8-41.2	52-57		21-53	14-31	9-13	2-4	1-6	2-7	6 (3)	4 (3)
	0.05-0.20	30 and 37	54 and 65	Mean . . .	3.2 (8)	10 (8)	90 (8)	0 (8)	46.5 (8)	59 (8)	SF (3), F (5)	16 (8)	30 (8)	7 (8)	4 (8)	1 (8)	38 (8)	4 (8)	
				Range . . .	0.8-10.7	0-37	73-100	0	37.0-59.0	52-64		0-53	8-38	2-17	2-9	0-2	17-33	1-7	1-7

Herit., hematocrit
F, fluid
Lymph., lymphocytes
R.C.H.C., mean corpuscular hemoglobin concentration
M.C.V., mean corpuscular volume
MBL., myeloblasts

Abbreviations
M Myel., metamyelocytes
MNC, mononuclear cells, i.e., lympho- and monocytes
Myelc., myelocytes
Normbl., normoblasts
PMN., polymorphonuclear granulocytes

PMN-EOS, polymorphonuclear eosinophils
PMN-N., polymorphonuclear neutrophils
S, solid
SF, semi-fluid
Retic., reticulocytes
W.B.C., white blood cell count

However, animals of group V within 9 days after elevation of daily dosage from 0.05 to 0.1 and 0.2 mgm. per kgm. showed advanced changes in blood and marrow consistent with the lesions found in the "acute" animals and in groups PI and PII.

2) *Lesions of the intestinal tract.* All animals had a natural oral mucosa and esophagus. However, the stomach and intestinal canal were filled in most cases with a yellow-brownish fluid and were often distended. Parts of the colon and rectum were spastically contracted. The fluid found in the gastro-intestinal tract was a transparent, viscid material, almost gelatinous in early stages but later more watery and plasma-like. Only in later stages were leucocytes and blood present.

Microscopic examination of tissues from small and large intestines, taken from animals as early as 6 and 12 hours after injection of 40 mgm. per kgm. of 4-amino-PGA, revealed venous hyperemia and marked dilatation of capillaries and venules in mucosa and submucosa. As a result the distal ends of villi were distended. Plasma extravasation was evident in the submucosa between the dilated vessels and the epithelium. The epithelium was enlarged partly by cytoplasmatic vacuolation. In some instances it had desquamated permitting plasma and lymph to flow into the lumen. After 24 and 48 hours these phenomena were more pronounced. Both surface and crypt epithelium showed, in addition to marked enlargement and vacuolation, extensive desquamation associated with rapid, often abnormal, regeneration of cells containing atypical, giant nuclei. Infiltration of villi and submucosa with neutrophils, lymphocytes and eosinophils began at 24 hours and, thereafter, increased progressively. Many polymorphonuclears mixed with desquamated epithelium could be found forming plugs in the crypts and were still present at a time when the marrow was almost completely free of granulocytes. After 72 hours both the small and large intestine showed extensive broadening and apparent shortening of villi or plicae due to extensive leucocytic infiltration, hyperemia, and edema. There were also small areas of fresh hemorrhages due to loss of the superficial parts of plicae.

In animals poisoned by chronic administration the extent of damage to the intestinal tissues paralleled the findings in bone marrow. Thus, groups PI and PII exhibited intestinal changes like those seen in animals of the "acute" group. Intestinal lesions were absent in Group I and only one of each of group II and III exhibited characteristic, early changes. Marked intestinal edema, desquamation of mucosa, and infiltration of leucocytes were noted in group IV. Finally, intestinal tracts of animals of group V were indistinguishable from those of experimental animals 72 hours after receiving 40 mgm. per kgm.

3) *The lymphoid system.* By the second and third days after the dose of 40 mgm./kgm. lymph nodes of the axilla, groin, and mesentery as well as lymph follicles of the spleen and lymph plaques of the intestine decreased moderately in size. At the same time a decreased number of lymphocytes was found in the circulation. Such changes might be related to migration of lymphocytes into the wall of the gut where they were found in abundance. It is pertinent to note that, with the exception of cortical pyknosis of the thymus in rats 48 and 72 hours after 40 mgm./kgm., necrotic changes in lymphoid tissues were not observed.

The relatively moderate effect on lymphoid tissues is one of the outstanding differences between the actions of 4-amino-PGA and those of nitrogen mustard (15).

4) *Other lesions.* Apart from the lesions described above, a general venous congestion of all internal organs was noted.

5) *Complications.* In five per cent of rats receiving 4-amino-PGA by chronic administration, salmonella infections altered the typical course of poisoning. After gut lesions were established, an ascending infection took place with enlargement of the mesenteric lymph nodes, abscess formation, multiple fibrinoid necrosis in liver, spleen, kidney and lung, bronchitis and bronchopneumonia.

Discussion. The derangements produced in rats by 4-amino-PGA include failure to gain weight, hypoplasia of bone marrow, and edema of the intestinal tract associated with desquamation and diarrhea. Each of these changes is found in folic acid deficiency and their combined appearance forms the classical syndrome (1). Nitrogen mustards and x-rays also produce lesions in bone marrow and intestinal tract but at the same time damage severely all lymphoid tissues. However, the fact that 4-amino-PGA has a more selective action in erythro- and myelopoietic tissues by comparison with its effects on lymphoid tissues is consistent again with the syndrome of folic acid deficiency (16). Moreover, intestinal changes following 4-amino-PGA and nitrogen mustard differ. Following fatal poisoning with nitrogen mustard the epithelium of the intestinal tract enlarges to a greater extent without desquamation and hyperemia and at the same time leucocytic infiltration of submucosa is not as marked as in 4-amino-PGA intoxication (17).

On the basis of the lesions produced by 4-amino-PGA, as described above, and its structural similarity to PGA it is reasonable to conclude that 4-amino-PGA acts as an antagonist of folic acid. This conclusion is supported by work on the growth of *Streptococcus fecalis* R (7, 9, 18). However, in mice (7), rats and chicks (9) 4-amino-PGA differs from an ideal metabolite-antagonist (5) in that its actions are not readily prevented or reversed by PGA. In this respect 4-amino-PGA is unlike x-methyl folic acid which acts as a competitive antagonist of PGA in rats, mice, and chicks (3, 4). In addition, the speed of onset and severity of lesions following administration of 4-amino-PGA exceeds by far the effect obtained with the same x-methyl antagonist. It is also to be noted that stomatitis found in rats receiving the reversible antagonist was missing in animals given 4-amino-PGA.

The discrepancies between the actions of 4-amino-PGA and those of an ideal metabolite-antagonist might be related to a marked affinity of the potent agent for the physiological loci of action of pteroylglutamic acid. On this basis the rapid onset and severity of lesions caused by the antagonist could be considered to result from an immediate and absolute folic acid deficiency in affected cells leading to their rapid degeneration and death. Accordingly the administration of folic acid might be relatively ineffective in preventing or reversing the toxic actions of 4-amino-PGA.

The failure to observe oral lesions in rats following administration of 4-amino-PGA corresponds to the findings in rats receiving folic acid deficient diets (16). However, such lesions are commonly found in patients treated with 4-amino-PGA

(11). Moreover, one might speculate that various antagonists of folic acid could differ sufficiently in distribution among the tissues of higher organisms such that at critical levels of dosage characteristic patterns of response could be expected.

SUMMARY AND CONCLUSIONS

1. The toxicity of 4-amino-pteroylglutamic acid (4-amino-PGA) has been studied in mice and rats following acute and chronic administration.

2. The agent was almost as toxic in mice and possibly more toxic in rats when given daily in fractions of the lethal dose.

3. The course of fatal intoxication was not altered by doses exceeding the LD_{50} at least tenfold.

4. The toxicity of 4-amino-PGA was reduced to only a limited extent in mice by simultaneous administration of large doses of either PGA or pteroyltri-glutamic acid.

5. The syndrome produced in rats corresponded to a folic acid deficiency and consisted of loss of weight, hypoplasia of bone marrow, and intestinal lesions with diarrhea. The syndrome was rapid in onset and led quickly to death.

6. It is concluded that 4-amino-PGA produces an absolute immediate deficiency of pteroylglutamic acid (PGA). The affected cells degenerate rapidly, which makes reversibility by PGA improbable. Regeneration can be expected only from cells relatively insensitive to the actions of the agent.

REFERENCES

1. JUKES, T. H., AND STOKSTAD, E. L. R.: *Physiol. Rev.*, **28**: 51, 1948.
2. MARTIN, G. J., TOLMAN, L., AND MOSS, J.: *Science*, **106**: 168, 1947.
3. FRANKLIN, A. L., STOKSTAD, E. L. R., AND JUKES, T. H.: *Proc. Soc. Exper. Biol. and Med.*, **65**: 368, 1947.
4. FRANKLIN, A. L., STOKSTAD, E. L. R., BELT, M., AND JUKES, T. H.: *J. Biol. Chem.*, **169**: 427, 1947.
5. WOOLEY, D. W.: *Physiol. Rev.*, **27**: 308, 1947.
6. SEEGER, D. R., SMITH, J. M., JR., AND HULTQUIST, M. E.: *J. Am. Chem. Soc.*, **69**: 2567, 1947.
7. FRANKLIN, A. L., STOKSTAD, E. L. R., AND JUKES, T. H.: *Proc. Soc. Exper. Biol. and Med.*, **67**: 398, 1948.
8. MINNICH, V., AND MOORE, C. V.: *Fed. Proc.*, **7**: 276, 1948.
9. OLESON, J. J., HUTCHINGS, B. L., AND SUBBAROW, Y.: *J. Biol. Chem.*, **175**: 359, 1948.
10. WOLL, E.: *Trans. N. Y. Acad. Sci., Ser. 2*, **10**: 83, 1948.
11. FARBER, S., DIAMOND, L. K., MERCER, R. D., SYLVESTER, R. F., AND WOLFF, J. A.: *New England J. Med.*, **238**: 787, 1948.
12. THIERSCH, J. B., AND PHILIPS, F. S.: To be published.
13. WINTROBE, M.: "Clinical Hematology", 2nd. Edition, Lea and Febiger, Philadelphia, 1946.
14. MILLER, L. C., AND TAINTER, M. L.: *Proc. Soc. Exper. Biol. and Med.*, **57**: 261, 1944.
15. KINDRED, J. E.: *Arch. Path.*, **43**: 253, 1947; GRAEF, I., KARNOFSKY, D., JAGER, V. B., KRICHESKY, B., AND SMITH, H. W.: *Amer. J. Path.*, **24**: 1, 1948.
16. ENDICOTT, K. M., DAFT, F. S., AND OTT, M.: *Arch. Path.*, **40**: 364, 1945; SPICER, S. S., DAFT, F. S., SEBRELL, W. H., AND ASHBURN, L. L.: *Public Health Reports*, **57**: 1559, 1942.
17. KARNOFSKY, D. A., THIERSCH, J. B., PATTERSON, P. A., AND RIDGWAY, L. M.: *Anat. Rec.*, **100**: 50, 1948.
18. SMITH, J. M., JR., COSULICH, D. B., HULTQUIST, M. E., AND SEEGER, D. R.: *Trans. N. Y. Acad. Sci., Ser. 2*, **10**: 82, 1948.

THE PROTECTIVE ACTION OF VARIOUS AGENTS AGAINST CHLOROFORM-EPINEPHRINE VENTRICULAR FIBRILLATION

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The production of fatal ventricular fibrillation in barbitalized dogs under chloroform anesthesia by the intravenous injection of large doses of epinephrine is a well established phenomenon (1).

It has been reported that certain agents presumed to produce coronary vasodilatation such as quinacrine (1) papaverine (2), or nitrites (3), the intravenous administration of procaine (4, 5, 6, 7), quinidine (8), or adrenolytic substances such as priscol (9, 10) and dibenamine (10, 11, 12) are useful in the prophylaxis of this phenomenon.

In the present series 3 groups of agents were tested for their protective influence against the chloroform-epinephrine effect on the dog's heart: a) coronary vasodilators: sodium nitrite (3 dogs), aminophylline (6 dogs), papaverine (3 dogs), and quinacrine (2 dogs).

b) those decreasing myocardial excitability: procaine (11 dogs), quinidine sulfate (6 dogs).

c) adrenolytic agents: priscol (4 dogs), dibenamine (2 dogs).

The production of ventricular fibrillation is not an invariable result of the administration of intravenous epinephrine during chloroform anesthesia to barbitalized dogs. For this reason, the absence of fibrillation alone cannot be used as an index of the protecting properties of the agent administered as a test drug.

In the present series, 21 dogs manifested ventricular fibrillation following the first or subsequent administrations of chloroform-epinephrine. Of these 21 dogs, 18 (85 per cent) demonstrated a tachycardia with increasing ventricular excitability immediately preceding the onset of fibrillation. For purposes of this study these changes are considered to represent "prefibrillation" changes. To protect properly against the onset of chloroform-epinephrine ventricular fibrillation, an agent should prevent the onset of "pre-fibrillation" changes as well as fibrillation.

METHOD. Medium sized dogs anesthetized with barbital 250 or 300 mgm. per kgm. were used. The common carotid artery was cannulated and the blood pressure recorded on moving photographic paper by means of a Hamilton optical manometer (13).

The drug to be tested was injected into an exposed femoral vein, and enough time allowed to elapse for the blood pressure and pulse rate to become stabilized following the injection. Chloroform was administered by means of a positive pressure anesthetic machine and face mask, the rate of flow regulated so as to minimize the fall in blood pressure as much as possible. Following 5 minutes of chloroform administration, epinephrine 0.02 mgm. per kgm. was rapidly injected into the femoral vein, and the chloroform immediately discontinued.

Tracings were obtained as follows: at the time the test drug was administered and continued until the maximum effect was manifest; at the onset of the chloroform inhalation

and at one-minute intervals during chloroform administration; and at the time of epinephrine injection, continuing until ventricular fibrillation ensued or a definite trend back toward normalcy had been established. Control tracings were made prior to the administration of the test drug in each instance.

The chloroform-epinephrine administration was repeated at regular intervals without further administration of the test drug in the majority of the dogs who survived the first administration.

RESULTS. Table I summarizes the maximum rhythm changes produced in each dog in the series and indicates the time intervals elapsing between the first and subsequent chloroform-epinephrine administrations.

1. Sodium nitrite, 10 mgm. per kgm. was given to 3 dogs (#16, 17, 18). In all cases there was an associated fall in blood pressure, but no appreciable change in cardiac rate or rhythm. Following the injection of epinephrine to dog #16 there was no increase in blood pressure but rhythm disturbances appeared as demonstrated by complete heart block with idio-ventricular rhythm followed by coupled ventricular premature contractions. Normal rhythm was restored after 4 minutes. The chloroform-epinephrine administration was repeated twice with identical results. Dogs #17 and 18 responded to the epinephrine injection with an abrupt rise in blood pressure and pulse rate. The tachycardia was followed by increased ventricular irritability in both cases, and dog #17 went on to fatal ventricular fibrillation. Dog #18 returned to a normal mechanism after 30 seconds of ventricular premature contractions. Repetitions of the chloroform-epinephrine administration to dog #18 up to 60 minutes produced similarly bizarre tracings. Neither dog #16 nor 18 went into ventricular fibrillation on any of the injections.

2. Aminophylline, 10 mgm. per kgm. was given to 6 dogs producing a fall in blood pressure in 4 (#9, 11, 12, and 13), and a rise in 2 (10 and 14). The cardiac rate and rhythm were unchanged. Following the epinephrine injection there was an abrupt rise in blood pressure and heart rate in all six. In 2 (#10 and 13) the tachycardia proceeded to ventricular fibrillation. In the remainder (#9, 11, 12, 14) there were many ventricular premature contractions lasting from 2 to 6 minutes. Repetition of chloroform-epinephrine administration to dog #12 produced ventricular fibrillation. Repetitions to dogs #9, 11, and 14 produced pre-fibrillation changes similar to those following the first administration. None of these 3 dogs developed ventricular fibrillation, even after an interval of $1\frac{1}{2}$ to 2 hours following the administration of the aminophylline.

3. Papaverine, 5 mgm. per kgm. was given to 3 dogs (#20, 21, 22). The administration was followed by an increase in pulse pressure due to a fall in diastolic pressure in all three. Following the epinephrine injection there was no significant rise in blood pressure in any case. Dog #20 demonstrated a tachycardia followed by ventricular premature contractions and fibrillation; dog #21 demonstrated a tachycardia followed immediately by ventricular fibrillation, and dog #22 had coupled ventricular premature contractions followed by fibrillation.

4. Quinacrine, 20 mgm. per kgm. was given to 2 dogs (#27 and 28). In both cases there was an associated fall in blood pressure. Following the ad-

TABLE I

Summary of maximum effect on rhythm following chloroform-epinephrine

DRUG	DOG	FIRST CHLOROFORM-EPINEPHRINE ADMINISTRATION			SUBSEQUENT CHLOROFORM-EPINEPHRINE ADMINISTRATIONS*		
		No irregularities (protection)	Pre-fibrillation changes. No fibrillation	Ventricular fibrillation	No irregularities (protection)	Pre-fibrillation changes. No fibrillation	Ventricular fibrillation
Chloroform - epinephrine alone with no protective substance	1		X			X	
	2			X			
	3			X			
Sodium nitrite 10 mgm./kgm.	16		X			XX	
	17			X			
	18		X			XXXX	
Aminophylline 10 mgm./kgm.	9		X			XX(1)	
	10			X			
	11		X			XX(2)	
	12		X				X(3)
	13			X			
	14		X			XXX(4)	
Papaverine 5 mgm./kgm.	20			X			
	21			X			
	22			X			
Atabrine 20 mgm./kgm.	27		X			X(5)	X(6)
	28		X				X(7)
Procaine 20 mgm./kgm.	4			X			
	6	X				XXXX(8)	
	7		X				X(9)
	15			X			
	29	X				X	X
	30		X			X	
	31			X			
	32			X			
	33			X			
	34			X			
	5			X			
Respiratory arrest following administration of procaine 25 mgm./kgm.							
Quinidine Sulfate 10 mgm./kgm.	25		X (3 sec. duration)				
	26	X			X		
	35		X			XX	
	36	X					X
	37	X				X	
	38	X			XXXX(10)		

TABLE 1—*Concluded*

DRUG	DOG	FIRST CHLOROFORM-EPINEPHRINE ADMINISTRATION			SUBSEQUENT CHLOROFORM-EPINEPHRINE ADMINISTRATIONS*		
		No irregularities (protection)	Pre-fibrillation changes. No fibrillation	Ventricular fibrillation	No irregularities (protection)	Pre-fibrillation changes. No fibrillation	Ventricular fibrillation
Priscol 10 mgm./kgm.	24	X			X		
	39	X			XX		
	40	X			XX		
	42	X					
Dibenamine 20 mgm./kgm.	23	X			X		
	41	X					

* At intervals of 15 minutes unless otherwise specified.

- (1) 20 and 35 minutes after initial administration.
- (2) 20, 90, and 110 minutes after initial administration.
- (3) 20 minutes after initial administration.
- (4) 20, 40, and 70 minutes after initial administration.
- (5) 10 minutes after initial administration.
- (6) 20 minutes after initial administration.
- (7) 10 minutes after initial administration.
- (8) 10, 30, 45, and 60 minutes after initial administration.
- (9) 20 minutes after initial administration.
- (10) 15 and 30 minutes, 1½, 2½, and 3½ hours after initial administration.

ministration of chloroform-epinephrine to dog #27 there was no hypertensive response, but a tachycardia was manifest followed by marked ventricular irregularity and A-V conduction changes. Repetition of these agents produced an abrupt rise in blood pressure, tachycardia, and ventricular fibrillation. Dog #28, following epinephrine, demonstrated a hypertensive response followed by ventricular premature contractions, ventricular tachycardia and tachysystole of 12 seconds duration. Ten minutes later, readministration of chloroform-epinephrine produced fibrillation.

5. Procaine, 20 mgm. per kgm. was given to 10 dogs with no associated change in rate, rhythm, or blood pressure. Following the chloroform-epinephrine administration there was an abrupt and significant rise in blood pressure in all cases. Six of the dogs developed ventricular fibrillation. Dogs #7 and 30 developed pre-fibrillation changes, with return to normal mechanism in 3 and 2 minutes, respectively. Dogs #6 and 29 responded to epinephrine with a sinus tachycardia demonstrating no irregularities in rhythm. Repetition of the chloroform-epinephrine administration to the dogs which had shown pre-fibrillation changes (#7 and 30) reproduced identical changes in #30, and produced ventricular fibrillation in #7. Readministration of the chloroform-epinephrine to dogs that had been protected (#6 and 29) produced marked pre-fibrillation changes, and a third administration to dog #29 resulted in ventricular fibrillation.

Dog #5 was given 25 mgm. procaine per kgm. and immediately after the injection died.

6. Quinidine sulfate, 10 mgm. per kgm. was given to 6 dogs, and was associated with a very slight fall in blood pressure and marked slowing of the rate in all. Following the epinephrine injection there was an abrupt rise in blood pressure of significant degree, and a rise in pulse rate to approximately the control level. There were no rhythm disturbances in 4 of the 6 dogs following the first chloroform-epinephrine administration. Dog #25 showed a very brief run of ventricular tachycardia lasting for 3 seconds with spontaneous reversion to the sinus mechanism. Dog #35 demonstrated definite pre-fibrillation changes following the first chloroform-epinephrine administration. The chloroform-epinephrine administration was repeated and produced ventricular fibrillation in 2 cases (#35 and 36), pre-fibrillation changes in 2 (#26 and 37), and no rhythm disturbances in 2 (#25 and 38). It should be noted that dog #25, which showed a 3-second run of ventricular tachycardia after the first chloroform-epinephrine administration was protected for the second. Dog #38 failed to show evidence of any rhythm disturbances up to $3\frac{1}{2}$ hours after having received quinidine.

7. Prisol, 10 mgm. per kgm. was given to 4 dogs with a marked rise in systolic pressure and moderate increase in rate. Epinephrine was followed by a slight rise in systolic and a significant fall in diastolic pressure, and a tachycardia in all cases. There were no irregularities in rhythm. Chloroform-epinephrine was repeated at 15 minute intervals once (#24) or twice (#39, 40) with identical results.

Dibenamine, 20 mgm. per kgm. was given to 2 dogs with essentially no changes in blood pressure and slight slowing of the rate. After an interval of 30 minutes chloroform-epinephrine administration produced a tachycardia in both, but no rhythm disturbances were noted. Re-administration of chloroform-epinephrine after 15 minutes produced identical results.

DISCUSSION. Of the 14 dogs receiving so-called "coronary vasodilators" 6 had ventricular fibrillation following the first chloroform-epinephrine administration, and the remaining 8 demonstrated pre-fibrillation changes of considerable severity. None were protected in the sense of preventing the changes which have been seen to immediately precede fibrillation. These findings are at variance with those of Melville (1, 3), who reported protection against chloroform-epinephrine ventricular fibrillation with coronary dilators.

The agents in the second group were variable. Procaine protected 2 dogs against arrhythmias following the first chloroform-epinephrine administration, 1 dog died following procaine administration alone, 2 showed marked pre-fibrillation changes and 6 manifested ventricular fibrillation. The protection of 2 out of 11 dogs, and the death due to procaine of 1 out of 11 dogs indicates that procaine is unreliable as a protective agent, findings in agreement with the conclusions of Wiggers and Wegria (4), and Nickerson (7), who found procaine to be of very little prophylactic value.

Quinidine sulfate in the dose employed had appreciable protective properties, a finding in agreement with Wegria and Nickerson (8). Four of the 6 dogs failed to show any of the arrhythmias after the first chloroform-epinephrine administration, which appeared following subsequent administrations in all but 1 (#38). One dog (#25) showed only a very transient pre-fibrillation change

which reverted to the sinus mechanism after only 3 seconds, changes which were insignificant for all practical purposes. Dibenamine and priscol, adrenolytic agents, gave complete protection to 2 and 4 dogs, respectively, finding in agreement with previous reports (8, 9, 10, 12).

It has been reported pro (14) and con (1, 11) that an abrupt and definite rise in blood pressure is essential to the production of this type of ventricular fibrillation. Twenty-seven of the dogs showed a rise in systolic pressure in excess of 20 mm. Hg, 8 of which showed no rhythm disturbances. Eight dogs demonstrated no appreciable hypertensive response (less than 20 mm. Hg), of which 5 had pre-fibrillation changes or fibrillation, and 1 dog had a fall in systolic pressure (10 mm. Hg) and fibrillated. From this evidence it is apparent that the pressor effect of epinephrine is not related to the production of ventricular fibrillation during chloroform inhalation.

SUMMARY

1. Three groups of agents were studied for their prophylactic effect against the production of ventricular fibrillation in barbitalized dogs given epinephrine during chloroform anesthesia.

a. So-called coronary vasodilating agents: sodium nitrite, aminophylline, papaverine, and quinacrine.

b. Agents decreasing myocardial excitability: procaine and quinidine sulfate.

c. Adrenolytic agents: dibenamine and priscol.

2. Criteria for protection against ventricular fibrillation were defined so as to include prevention of increased ventricular excitability preceding fibrillation as well as prevention of fibrillation.

3. Coronary dilating agents afford no protection.

4. Procaine affords no practical protection.

5. Quinidine sulfate affords appreciable and consistent protection.

6. Adrenolytic agents afford complete protection.

7. A pressor response to epinephrine is not necessary for the production of this type of ventricular fibrillation.

REFERENCES

1. MELVILLE, K. I.: *THIS JOURNAL*, 87: 350, 1946.
2. MELVILLE, K. I.: *THIS JOURNAL*, 66: 107, 1939.
3. MELVILLE, K. I.: *THIS JOURNAL*, 94: 136, 1948.
4. WIGGERS, C. J., AND WEGRIA, R.: *Am. J. Physiol.*, 131: 206, 1940.
5. BURSTEIN, C. T., AND MARANGONI, B. A.: *Proc. Soc. Exper. Biol. and Med.*, 43: 210, 1940.
6. BITTRICH, N. M., AND POSERS, W. F.: *Cur. Res. Anes. and Anal.*, 27: 181, 1948.
7. NICKERSON, M.: *Fed. Proc.*, 7: 247, 1947.
8. WEGRIA, R., AND NICKERSON, N. D.: *THIS JOURNAL*, 75: 50, 1942.
9. AHLQUIST, R. P., HUGGINS, R. A., AND WOODBURY, R. A.: *THIS JOURNAL*, 89: 271, 1947.
10. NICKERSON, M., AND GOODMAN, L. S.: *Fed. Proc.*, 7: 397, 1948.
11. GARB, S., AND CHENOWETH, M. B.: *Fed. Proc.*, 7: 220, 1948.
12. GARB, S., AND CHENOWETH, M. B.: *THIS JOURNAL*, 94: 12, 1948.
13. HAMILTON, W. F., BREWER, G., AND BROTMAN, I.: *Am. J. Physiol.*, 107: 427, 1934.
14. SHEN, T. C. R., AND MORRI, R.: *Arch. Internat. Pharmacodyn. et de Therap.*, 64: 58, 1940.

EFFECTS OF MORPHINE, CODEINE, AND DILAUDID ON BLOOD FLOW

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After the administration of morphine vomiting occurs regularly in the dog and nausea and vomiting occasionally in man. This action of morphine has been demonstrated to be central in origin and the result of a preliminary "stimulating" action on the vomiting center (1). Not so commonly recognized is the fact that after depression of the vomiting center has occurred, the assumption of an upright position may again precipitate nausea and vomiting. This has been observed to occur in the human with 0.3 to 0.5 mgm./kgm. of morphine (2). The fact that nausea and vomiting did not occur while in the horizontal position but that it could be repeatedly produced in the same individual on standing suggested the possibility that these phenomena were of circulatory origin.

METHODS. Dogs were anesthetized with sodium barbital 250 mgm./kgm. intraperitoneally. The blood pressure was obtained from the right carotid artery using an optical manometer (3) and blood flow to the leg or head was measured with a Gregg-Shipley rotameter (4). Chlorazol Fast Pink, 2 cc. of an 8 per cent solution per kgm. was used as the anticoagulant. For controls, simultaneous blood pressures and rate of flow were determined in the horizontal position and then the head or legs suddenly raised to an angle of approximately 45°. The drug was then given either intra-arterially (i.a.) into the distal arm of the flow meter or intravenously (i.v.) into the femoral vein and at varying intervals up to two hours blood flow and pressures were obtained in the horizontal position and at a 45° angle. In every dog but two, only one drug was used. The use of morphine after codeine, (LF 5), and after dilaudid, (LF 8A), were the two exceptions.

RESULTS. The elevation of either the head or hind legs of the dogs to a 45° angle during the control period had no detectable effect on either blood pressure or flow.

Morphine and codeine in the concentrations used when administered i.a. produced a marked increase in flow usually followed by a drop in pressure in both the head and leg regions. In measurements of head flow for example: codeine 0.1 mgm./kgm. increased the flow by 21 cc. and lowered the pressure from an average of 181/143 to 178/140 for about a minute and a half. This drop in pressure, while small occurred in all dogs receiving codeine. Morphine, 0.1 mgm./kgm., produced a greater increase in flow and fall in pressure than did codeine. The flow increased to a maximum of 27 cc. and the pressure fell to 143/98 from 190/148. The pressure with both morphine and codeine returned to the original level in 2-3 minutes. The flow returned toward normal in about the same period of time although in a few dogs the flow remained at a level of from 5-10 cc./min. above the control level for varying periods up to an hour. The same general response was observed with the leg although the absolute flow

of course was less. The blood pressure and flow response to 0.01 mgm./kgm. of dilauid is more like codeine. The drop in pressure is even smaller or may not occur and the increase in flow less, the increase averaging 10 cc. and lasting about a minute to a minute and a half.

Tables I and II show clearly that with morphine, but not with codeine or dilauid, there is a significant drop in flow to either the head or leg region if

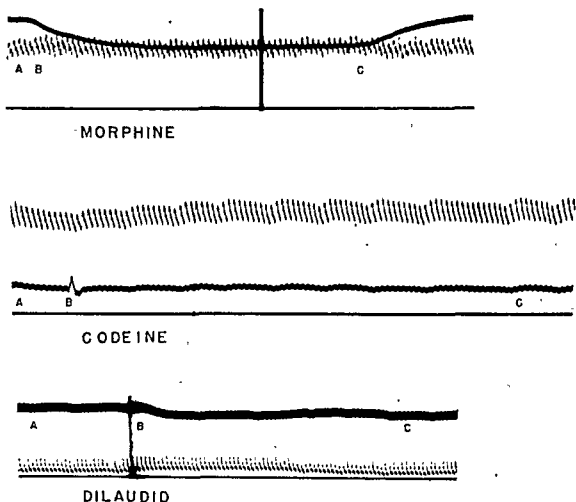


FIG. 1. A, CONTROL, DOG IN HORIZONTAL POSITION. B, HEAD ELEVATED 45°. C, RETURNED TO HORIZONTAL

that part is elevated 45°. Dog HF 17 was the sole exception. The decrease in flow occurs whether the morphine is given i.a. or i.v. Further, once this response becomes evident, increasing the amount of morphine administered does not increase the drop in flow. With dog HF 11 a drop in flow of 13 cc./min. occurred with 0.1 mgm./kgm. and after 0.3 mgm./kgm. the decrease in flow was still 13 cc./min. Typical results are shown in fig. 1.

Lastly, dog LF 8A after receiving dilauid with no apparent effect of blood flow on elevation was given 0.1 mgm./kgm. of morphine, and on being raised 45° there was an immediate drop in flow of 19 cc./min. The same thing was observed with dog LF 5 who had received 1 mgm./kgm. of codeine and then 0.1 mgm./kgm. of morphine. The blood flow was reduced by 8 cc./min.

TABLE I
Head flow—cc./min.

DOG	DRUG	DOSE <i>mgm./kgm.</i>	CONTROL	RAISED 45°	HORIZONTAL
HF 8	Morphine	0.1	102	71	99
			99	68	98
HF 9	Morphine	0.3	46	30	50
			50	40	49
HF 10	Morphine	0.1	27	23	30
HF 11	Morphine	0.1	57	44	60
		0.1	64	34	67
		0.1	60	47	67
HF 17	Morphine	0.5	60	58	60
HF 18	Morphine	0.1	64	56	64
		0.1	72	56	73
			90	86	94
		0.3	62	54	68
			64	58	60
HF 12	Codeine	0.1	50	46	46
			38	41	38
		0.1	64	60	67
HF 12A	Codeine	1	32	30	36
HF 15	Dilaudid	0.01	78	79	79
		0.01	82	80	81
HF 16	Dilaudid	0.01	68	66	68
HF 16A	Dilaudid	0.01	69	70	70

TABLE II
Leg flow—cc./min.

DOG	DRUG	DOSE <i>mgm./kgm.</i>	CONTROL	RAISED 45°	HORIZONTAL
LF 3	Morphine	0.1	30	14	29
			34	23	28
LF 4	Morphine	0.1	31	22	30
			30	21	29
LF 4A	Codeine	1	35	34	36
LF 5	Codeine	1	24	22	25
	Morphine	0.1	26	18	24
LF 8	Dilaudid	0.01	59	56	61
			51	51	55
LF 8A	Dilaudid		62		60
			5		74

In the majority of dogs there was no effect on blood pressure when the flow decreased. A few showed a slight increase or decrease in blood pressure but never more than 3-4 mm. Hg.

Discussion:

That morphine has an antiemetic effect in dogs has been shown by Leake (5). In demonstrating this action, doses between 6 and 10 mgm./kgm. were used while the dosage range in these experiments was between 0.1 and 0.5 mgm./kgm. The antiemetic action of morphine does not occur within the dosage range used here.

These data suggest that morphine interferes with the vascular compensatory mechanism that normally insures an adequate cerebral blood flow during postural changes. This inhibition only becomes evident on elevation of the dog and is shown by the inability of the circulatory system to compensate for the change in position and the result is a reduced flow to the elevated part. With the reduced minute volume occurring with morphine and the drop in flow which occurs if the head is elevated the anoxia resulting might be sufficient to "stimulate" the vomiting center.

It would be expected, if this is true, that dilaudid and codeine would be less likely to produce vomiting because the vasoconstrictor center is not inhibited as shown by the ability of the circulatory system to maintain flow to the elevated part.

SUMMARY

1. Elevation of the hind legs or head of a dog to 45° after the administration of morphine causes a significant reduction in blood flow to the part raised.
2. Morphine may produce a partial inhibition of the vasoconstrictor center.
3. Codeine and dilaudid lack this effect.

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REFERENCES

1. HATCHER, R. A., AND WEISS, S.: *THIS JOURNAL*, 22: 139, 1923.
2. HANDLEY, C. A.: Unpublished data.
3. HAMILTON, M. F., BREWER, G., AND BROTHMAN, J.: *Am. J. Physiol.*, 107: 427, 1934.
4. GREGG, D. E., SHIPLEY, R. E., ECKSTEIN, R. W., ROTTA A., AND WEARN, J. T.: *Proc. Soc. Exper. Biol. and Med.*, 49: 267, 1942.
5. LEAKE, C. D.: *THIS JOURNAL*, 20: 359, 1922-23

ANESTHETIC PROPERTIES OF SODIUM 5-ALLYL-5-(1-METHYLBUTYL)-2-THIOBARBITURATE (SURITAL) AND CERTAIN OTHER THIOBARBITURATES IN DOGS¹

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Certain inherent disadvantages are common to all of those few barbiturates (Evipal, thiopental, Thioethamyl, Kemithal) which are detoxified with sufficient rapidity to make them useful for intravenous administration as anesthetic agents. Outstanding among these disadvantages are: (a) detoxication mechanisms become saturated if anesthesia is prolonged, so that these agents soon cease to retain their short acting characteristics and cumulative effects are noted; (b) certain reflexes, notably those having to do with the larynx, persist and are troublesome even in deep anesthesia; (c) respiration is very often seriously depressed; (d) these compounds, as is common to all barbiturates, lack the capacity to block specifically the central thalamic reception of painful stimuli; (e) it is generally believed that these compounds have an adverse effect upon cardiac activity.

The present study (1, 2, 3) is in search of an agent which might possess advantages over those compounds now in common use, particularly thiopental (Pentothal).

After a preliminary survey of several compounds which were made available to us by Dr. Bywater of the Parke, Davis and Company Research Laboratories, three were selected for further evaluation: sodium 5-allyl-5-(1-methylbutyl)-2-thiobarbiturate (Surital), sodium 5-ethyl-5-isoamyl-2-thiobarbiturate (Thioethamyl), and sodium 5-isopropyl-5-(2-methyl-2-pentenyl)-2-thiobarbiturate (B-10). See table I.

METHOD. With the exception of thiopental, which was used as the commercial sodium salt, these compounds were obtained as the acids and dissolved by neutralization with equivalent amounts of sodium hydroxide and buffered with fifty mgm. of sodium carbonate per gram of acid. Solutions of these various thiobarbiturates were used in concentrations between 0.75 and 5.0 per cent, calculated as acid weight.

In all experiments, except for the laryngeal spasm studies carried out on cats, the dog was used as the experimental animal. Administration in all instances was by vein and injection was made at a constant and uniform rate. Careful observations of the signs of anesthesia were recorded. The duration of anesthesia was arbitrarily fixed as the time from the onset of unconsciousness until the animal was able to stand erect when stimulated.

In the initial experiments all compounds were administered at a dose of 25 mgm./kgm. Sodium Thioethamyl was repeated at 50 mgm./kgm. because of its ineffectiveness at the lower dose level. Sodium B-10, being more potent, was used at a dose of 12.5 mgm./kgm.

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After evaluation of the potency at the same dose level, namely 25 mgm./kgm., sodium thiopental and sodium Surital were administered to dogs in their respective ratios of potency, namely 1.0 to 1.5. The doses used were 10 mgm./kgm. for Surital and 15 mgm./kgm. for thiopental.

In order to evaluate the cumulative potentialities of these thiobarbiturates, small doses of each compound were injected at hourly intervals. Dosage of the several drugs was adjusted to produce approximately the same duration of anesthesia with the first injection.

The effect of three thiobarbiturates, Surital, Thioethamyl, and B-10, upon the laryngeal reflex of the cat was determined according to the method of Burstein and Rovenstine (4). An attempt to produce laryngeal spasm in the dog by rectal dilatation was unsuccessful.

RESULTS. Quality of Anesthesia. Thiopental produced a smooth, rapid induction rarely accompanied by signs of stimulation or excitation. Satisfactory surgical anesthesia was produced with doses of 15 to 25 mgm./kgm. Emergence was fairly prompt and usually unaccompanied by excitation.

Surital effected an equally smooth induction, perhaps more rapid and with fewer signs of excitation. Doses of 10 to 20 mgm./kgm. resulted in good surgical

TABLE I
Thiobarbituric acid derivatives

		R ₁	R ₂
$ \begin{array}{c} \text{H}-\text{N}-\text{C}=\text{O} \\ \quad \\ \text{S}=\text{C} \quad \text{C} \\ \quad \\ \text{H}-\text{N}-\text{C}=\text{O} \end{array} $	Thiopental	$-\text{CH}_2-\text{CH}_3$	$-\text{CH}(\text{CH}_3)-\text{CH}_2-\text{CH}_2-\text{CH}_3$
	Surital	$-\text{CH}_2-\text{CH}=\text{CH}_2$	$-\text{CH}(\text{CH}_3)-\text{CH}_2-\text{CH}_2-\text{CH}_3$
	Thioethamyl	$-\text{CH}_2-\text{CH}_3$	$-\text{CH}_2-\text{CH}_2-\text{CH}(\text{CH}_3)_2$
	B-10	$-\text{CH}(\text{CH}_3)-\text{CH}_3$	$-\text{CH}_2-\text{C}(\text{CH}_3)=\text{CH}-\text{CH}_2-\text{CH}_3$

anesthesia. Emergence was similar to that observed with thiopental but was somewhat more rapid.

With Thioethamyl induction was characterized by more marked and frequent stimulation as compared with thiopental and Surital. In addition Thioethamyl did not produce satisfactory muscular relaxation and emergence was slow with prolonged drowsiness and muscular weakness.

B-10 produced an undesirable amount of stimulation during induction although the type of anesthesia was quite adequate. Emergence was rapid, but the animals frequently showed much stimulation, irritability and excitation which often persisted for fifteen to thirty minutes.

Anesthetic Potency. The essential data concerning the duration of anesthesia with a given dose are shown in tables II and III. The approximate ratio of potency (thiopental = 1) of the four thiobarbiturates studied is thiopental, 1.0; Surital, 1.5; Thioethamyl, <0.5; and B-10, >1.5.

Cumulative Effect. Table IV compares the cumulative effects of the four thiobarbiturates. Injections were stopped when one or more dogs in any one series showed a duration of anesthesia exceeding sixty minutes. In each series every injection produced anesthesia of longer duration than the previous injection. Thioethamyl produced the most marked cumulative effect, followed by thiopental and lastly by Surital and B-10. Figure 1 depicts graphically the

increases of duration of anesthesia over those of the initial injection. A statistical analysis of the differences in third hour durations (following the fourth injection) is shown in table V.

Laryngeal Spasm. All four thiobarbiturates produced sneezing, hiccoughing, and coughing (supposedly characteristic of laryngeal spasm) in cats to much the same degree. There does not seem to be any apparent advantage in the use of one particular drug.

TABLE II
Durations of anesthesia

DRUG	CONCENTRATION OF SOL'N	DOSE (ACID WT.)	NO. OF DOGS	AV. WT. OF DOGS	MEAN DURATION OF ANESTHESIA	S.E.M	t-VALUE
	%	mgm./kgm.		kgm.			
Sod. thiopental.....	5.0	25.0	22	8.8	74.4	7.1	10.47
Sod. Surital.....	2.5	25.0*	24	9.8	132.2	10.9	12.12
Sod. Thioethylamyl.....	2.5	25.0	5	8.9	15.4	4.7	3.27
Sod. Thioethylamyl.....	5.0	50.0	5	10.3	94.2	18.9	4.98
Sod. B-10.....	2.5	12.5	8	11.7	58.5	8.1	7.22

* Artificial respiration was necessary with a few animals to carry over an initial period of apnea.

TABLE III
Quality of anesthesia under equivalent doses of thiopental and Surital

DRUG	CONC. OF SOL'N	DOSE (ACID WT.)	NO. OF DOGS	AV. DURATION OF CORNEAL AREFLEXIA	AV. DURATION OF ANESTHESIA	COMMENTS
	%	mgm./kgm.		min.	min.	
Sod. thiopental...	3.0	15.0	8	8.5	26.0	Some restlessness on induction and emergence
Sod. Surital.....	2.0	10.0	8	7.3	26.6	No stimulation. Emergence and induction more rapid. The quality and depth of anesthesia equal

DISCUSSION. The quality of anesthesia following intravenous administration of Surital in dogs is as satisfactory as that obtained with thiopental. As a result of undesirable reactions during induction and emergence Thioethylamyl and B-10 are much less suitable for intravenous anesthesia than either thiopental or Surital. In addition, as will be discussed in subsequent paragraphs, Thioethylamyl requires a larger dose because of its low level of potency and exhibits a marked cumulative effect.

A comparison of potency based on the duration of anesthesia assigning thiopental a value of 1.00 shows Surital with a potency of about 1.5 and Thioethylamyl <0.5. Kelly, Shideman and Adams (5) observed results which were of the same

general order when the blood level at the time of return of righting reflexes was used as an index of potency; Surital having a value of 1.39 and Thioethamyl 0.57.

TABLE IV

Comparative cumulative action of four thiobarbiturates as measured by increases in duration of anesthesia following administration of each compound in fixed dosage at hourly intervals.

Increment in duration of anesthesia is expressed as percentage of initial anesthesia time.

DRUG	DOSE (ACTID WT.)	NO. OF DOGS	DURATION OF ANESTHESIA					
				Hour				
				0	1	2	3	4
Sodium thiopental	7.5 <i>mgm./ kgm.</i>	12	Minutes: Per cent of 0-hour time	7.6 ± 1.1 100	14.8 ± 2.0 195	31.5 ± 2.3 414	78.3 ± 8.1 1029 ± 106 (^t value = 9.6)	
Sodium Surital	5.0	16	Minutes: Per cent of 0-hour time	5.8 ± 1.6 100	6.5 ± 1.1 112	11.3 ± 1.5 195	22.5 ± 3.8 386 ± 64 (^t value = 6.0)	41.5 ± 9.3 716
Sodium Thio- ethamyl	12.5	5	Minutes: Per cent of 0-hour time	2.4 ± .24 100	8.4 ± 1.2 350	28.8 ± 4.5 1200	67.4 ± 9.7 2808 ± 474 (^t value = 5.9)	
Sodium B-10	3.75	7	Minutes: Per cent of 0-hour time. ...	6.3 ± 1.1 100	10.1 ± 2.4 160	22.6 ± 7.2 358	33.0 ± 5.7 523 ± 81 (^t value = 6.4)	45.8 ± 9.8 722

TABLE V

Statistical significance of the differences in third hour durations of anesthesia on basis of comparison of per cent increases of the third hour values over the 0-hour values

DRUG	SOD. PENTOTHAL	S. SURITAL	S. THIOETHAMYL	S.B.-10
Sod. Thiopental.....	—	5.16	3.65	3.77
Sod. Surital.....	5.16*	—	5.06	1.32†
Sod. Thioethamyl....	3.65	5.06	—	4.74
Sod. B-10.....	3.77	1.32†	4.74	—

* All values are 'values of the differences between respective means.

† The Sod. B-10 and Sod. Surital series' differences are not statistically significant.

From an inspection of figure 1 and table IV it is quite apparent that Surital exhibits a lower rate of accumulation than either thiopental or Thioethamyl. This is undoubtedly due to the greater inherent potency of Surital since as shown

by Kelly *et al.*, (5) the shape and slope of the blood level curves for all three drugs is similar indicating that the body detoxifies each compound at the same rate irrespective of its absolute potency as an anesthetic. The rate of cumulation is undoubtedly a function of the total dose administered, the more potent drugs requiring a smaller dose and hence a shorter time for detoxication. In addition, there may be degradation products which may either interfere with detoxication or possess a depressant action in their own right. Recently Shideman, Kelly and Adams (6), using the spectrophotometric method, followed blood levels

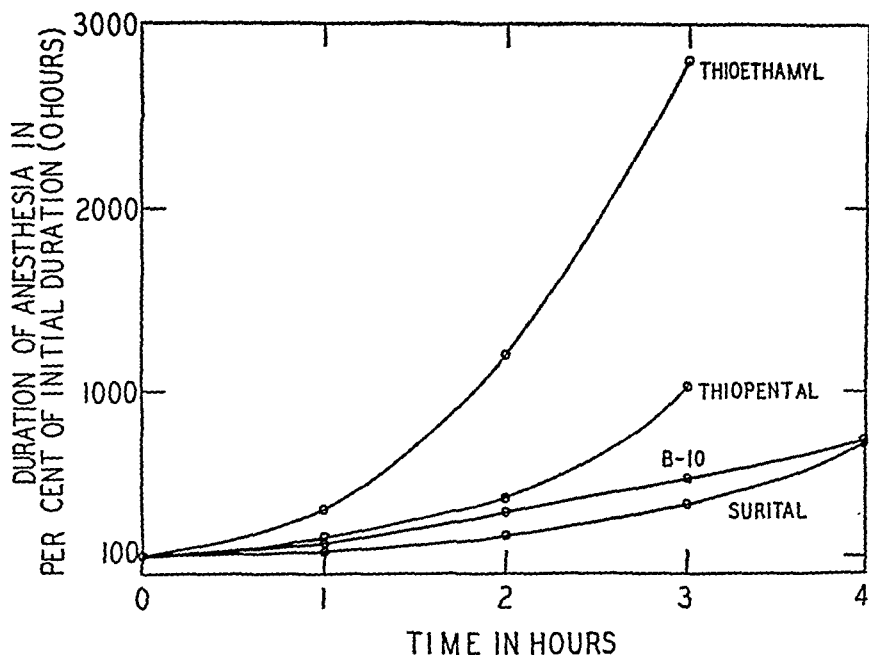


FIG. 1. CUMULATIVE ACTION OF HOURLY INJECTIONS

The time values on the abscissa represent the hour on which the injections were administered. Doses administered each hour are listed in table IV.

of thiopental in dogs receiving repeated doses of the drug at short intervals. The plasma level at which the righting reflexes returned was higher with each successive dose. This would indicate the development of an acute tolerance. However it is possible that degradation products, in addition to thiopental, were measured by the method and that as more and more breakdown products accumulated "apparent" higher values for thiopental were observed.

In conclusion it may be stated that Surital possesses certain advantages over some of the other more common intravenous anesthetic agents. It exhibits high potency, rapid induction and emergence with a few signs of stimulation, and produces satisfactory muscular relaxation. Respiratory depression is no greater than that observed with thiopental when both are given in equivalent anesthetic doses (3). The lower doses necessary with Surital should place less

strain upon detoxication mechanisms, allow for shorter post-operative sleeping time, and reduce the cumulative effect. Finally Surital shows low cardiac toxicity (3).

SUMMARY

The anesthetic properties of four thiobarbiturates, thiopental, Surital, Thioethamyl, and B-10, were compared in dogs. The quality of anesthesia was most satisfactory with Surital and thiopental. The ratio of potency, based upon the duration of anesthesia and using thiopental as a standard of 1.0, was Surital 1.5, Thioethamyl, <0.5 and B-10, >1.5 .

Surital exhibited considerably less cumulative effect than either thiopental or Thioethamyl and possesses other characteristics noted above which indicate it to be worthy of clinical trial. B-10 showed cumulative action of much the same degree as Surital but because of undesirable side-reactions was not considered to be a good intravenous anesthetic agent for the dog.

Based upon these observations, and the assumption that all thiobarbiturates are detoxified in a similar manner, it seems clear that further work should center on a search for a more potent agent in order to permit a greater duration of anesthesia without serious cumulative effects.

REFERENCES

1. WYNGAARDEN, J. B., WOODS, L. A., AND SEEVERS, M. H.: *Fed. Proc.*, 6: 388, 1947.
2. WYNGAARDEN, J. B., WOODS, L. A., RIDLEY, R., AND SEEVERS, M. H.: *Fed. Proc.*, 6: 388, 1947.
3. WOODS, L. A., WYNGAARDEN, J. B., RENNICK, B., AND SEEVERS, M. H.: *Fed. Proc.*, 6: 387, 1947; *THIS JOURNAL*, succeeding article.
4. BURSTEIN, C. L., AND ROVENSTINE, E. A.: *THIS JOURNAL*, 63: 42, 1938.
5. KELLY, A. R., SHIDEMAN, F. E., AND ADAMS, B. J.: *Fed. Proc.*, 7: 233, 1948.
6. SHIDEMAN, F. E., KELLY, A. R., AND ADAMS, B. J.: *Fed. Proc.*, 7: 255, 1948.

CARDIOVASCULAR TOXICITY OF THIOBARBITURATES: COMPARISON OF THIOPENTAL AND 5-ALLYL-5-(1-METHYLBUTYL)-2-THIOBARBITURATE (SURITAL) IN DOGS¹

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Evidence and opinion is divided as to the primary cause of death from thiobarbiturates. It is well established, of course, that both thiobarbiturates and their oxygen analogues can produce respiratory failure. Certain investigators believe that thiopental (Pentothal) and other similar derivatives possess a primary cardiotoxic action sufficient to cause death. This belief is predicated upon evidence obtained from the dog as follows: (a) additive toxic effects with digitalis (1); (b) sudden fall in arterial pressure and appearance of ectopic beats (2); (c) conduction disturbances with cardiac arrhythmia (3). Other investigators have been unable to establish a primary cardiotoxic action of thiobarbiturates. For example, Kohn and Lederer (4) found that thiopental did not produce primary cardiac death or ventricular fibrillation in the dog. They attributed all deaths to respiratory failure. Betlach, working with dogs (5), and Volpitto and Marangoni (6) in humans, found no significant changes in the electrocardiograms under thiopental anesthesia.

In view of the contradictory nature of previous studies, the experiments described herein (7) were designed to obtain if possible a definitive answer to this question by determining the direct cardiovascular toxicity of two thiobarbiturates by (a) means of the Starling heart-lung preparation, (b) comparison of lethal doses and types of death in animals spontaneously respiring with others respired artificially in order to control the factor of anoxia.

A. Heart-Lung Preparation. Ten experiments were performed using the Kraymer-Mendez (8) modification of the Patterson-Starling heart-lung preparation, with the exception that the venous inflow pump and coronary artery cannula were not used. Also the Stolnikow stromuhr was employed instead of the Weese variety. The cardiac output and arterial resistance were adjusted at the outset to give a standard work value for all experiments.

Both thiopental and Surital were employed as the sodium salts in one per cent solution (acid weight). All injections were made into the superior vena cava. In a given experiment two and one-half cc. (25 mgm.) of the given thiobarbiturate were injected every fifteen minutes until failure ensued. Also, for purposes of direct comparison in the same preparation, in most experiments one (1) injection of the alternate thiobarbiturate was substituted for one of the drug being principally studied.

Continuous measurements of right auricular pressure, arterial pressure, and heart volume were recorded on the kymograph drum. Periodic measurements of the cardiac output (left ventricular output minus the coronary artery flow), and heart rate were recorded.

Increased right auricular pressure and decreased cardiac reserve were used as criteria of heart failure.

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B. Intact Animal Experiments. Twelve experiments were carried out in dogs to evaluate the response of the cardiovascular system to thiopental and Surital. In all preparations arterial pressure was recorded from the carotid artery, and the trachea was cannulated to facilitate respiration. Injections were made into the femoral vein. Electrocardiographic records were obtained in several experiments. The animals were anesthetized using a minimum amount of the particular thiobarbiturate to be studied.

Six of the twelve animals were studied under spontaneous respiration and in these abdominal and thoracic pneumographic tracings were recorded. The other six dogs were respired artificially with positive intratracheal insufflation.

Thiopental and Surital were used as the sodium salts both with intermittent injection at regular intervals and also continuous intravenous infusion. The drugs were used in amounts inversely proportional to their anesthetic potency (9), i.e., 1.5 times as much thiopental, by weight or concentration, as Surital.

RESULTS. *A. Direct Cardiac Effect.* (1) *Acute rise in right auricular pressure.* One of the actions of these drugs measurable in the heart-lung preparation is the rise in right auricular pressure immediately following the injection of the drug into the superior vena cava. This increase in pressure extends over a period of a few seconds to a few minutes. The right auricular pressure, following the acute rise, dropped but usually did not reach the pressure level before the injection. Occasionally with Surital, a fall below the initial level followed the acute rise in pressure. In one instance there resulted only a drop in auricular pressure. One injection of the alternate drug, Surital, was carried out in the five experiments of the thiopental series and one injection of the alternate drug, thiopental, was made in each of two of the experiments of the Surital series. For each series there is plotted in figure 1 the average acute right auricular pressure rise in mm. against the number of injections. The alternate drug is represented by a separate point. It was thus possible to compare responses of the same heart-lung preparation to each drug. Whether one injection of Surital is given in a series of thiopental injections or thiopental is given in a series of Surital injections, the acute right auricular pressure rise produced by Surital is less than that produced by thiopental, and occasionally Surital produced a fall in pressure on its first few injections.

(2) *Decreased cardiac reserve.* The normal heart-lung preparation responds to increased cardiac load (elevation of the venous blood reservoir) with increased cardiac output with the result that there occurs only a slight increase in right auricular pressure. The term cardiac reserve is frequently given to the capacity of the heart described above. Thus decreased cardiac reserve is measurable by recording the greater increase in right auricular pressure in response to elevation of the venous blood reservoir.

Right auricular pressure responses to increased cardiac load were analyzed in the following manner:

Index of cardiac reserve (ICR)

$$= \frac{\text{Elevation of reservoir (mm.)} - \text{Rt. auric. pressure rise (mm.)}}{\text{Elevation of reservoir (mm.)}} \times 100$$

The theoretical 100 per cent cardiac reserve would be present only if the elevation of the venous blood reservoir produced no rise of the right auricular pressure. Since such a rise always occurs, the control ICR was equated to 100 per cent reserve for that particular preparation, and subsequently determined ICR values were

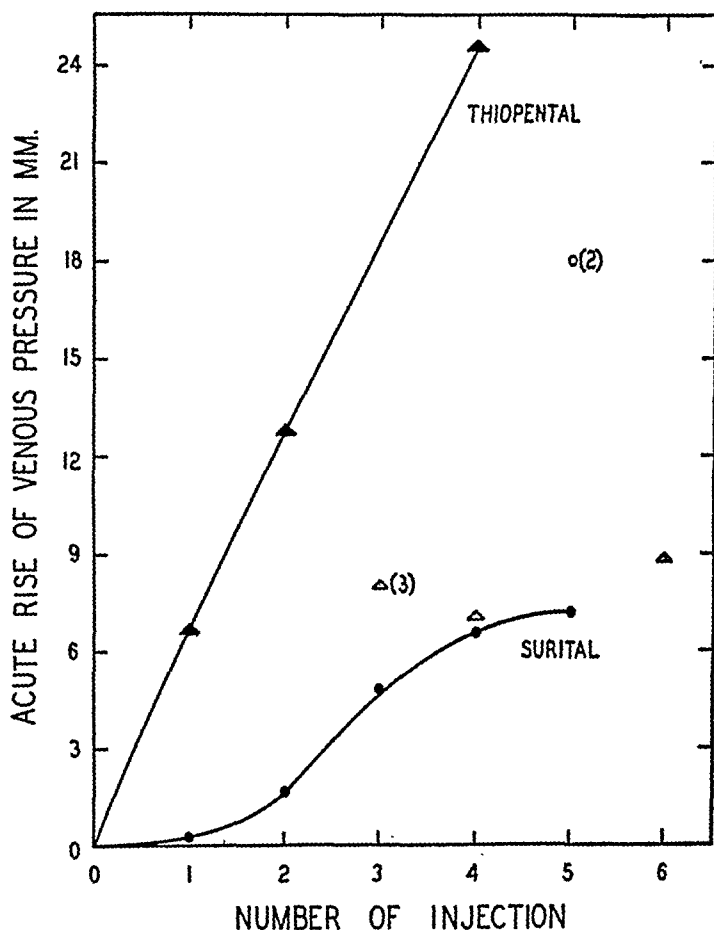


FIG. 1. ACUTE CARDIAC TOXICITY OF SURITAL AND THIOPENTAL

Points on the curves represent average immediate right auricular pressure responses to injections of the respective drug. The open triangles represent responses to substituted injections of Surital in the pentothal series. The open circle represents the responses to substituted injections of pentothal in the Surital series. The figures in parentheses represent the number of substituted injections whose average gave the point plotted. The points not thus marked represent one determination.

compared with this value in per cent of the initial control ICR figure. Thus, the per cent reserve equals $(\text{Determined ICR} \div \text{Control ICR}) \times 100$. The theoretical number of injections (see table I) required to produce 50 per cent cardiac reserve in each heart-lung preparation was determined from figure 2, A and B. From the number of injections the weight in mgm. of the drug required to

TABLE I

Comparison of cardiotoxicities of thiopental and surital in heart-lung preparation*

DRUG	EXPER. NO.	NO. OF INJ. (25 MGM. EA.) REQUIRED TO PRODUCE 50% REDUCTION OF CARDIAC RESERVE	WEIGHT OF VENTRICLES (UNCORR.)	DOSE AT 50% REDUCTION IN MGM /GRAM OF VENTRICLE	MEAN DOSE \pm S.E.m	t VALUE OF:	
						Mean	Difference of means
Surital	I	5.61	grams 81.0	1.73	1.85 \pm 0.31	5.97	1.11
	II	10.55	87.0	3.03			
	III	4.30	61.0	1.76			
	IV	4.52	76.5	1.48			
	V	4.90	95.2	1.29			
Pentothal	I	5.60	83.9	1.67	1.47 \pm 0.16	9.19	
	II	6.05	81.0	1.86			
	III	3.30	87.0	0.94			
	IV	4.60	85.8	1.34			
	V	4.00	66.1	1.53			

* As determined by right auricular pressure rises with successive injections.

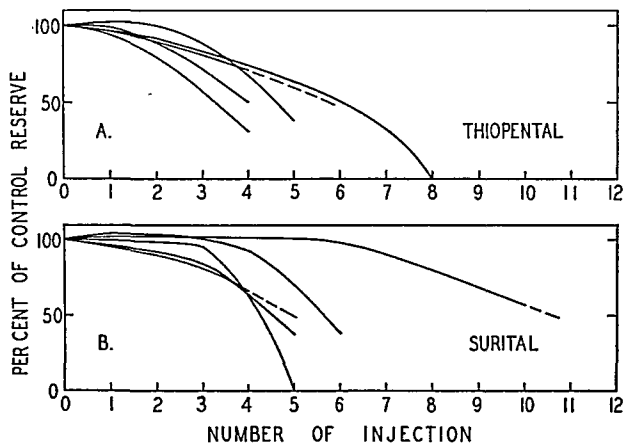


FIG. 2. RATES OF IMPAIRMENT OF CARDIAC RESERVE OF HEARTS UNDER SURITAL AND THIOPENTAL, ON BASIS OF RIGHT AURICULAR PRESSURE INCREASE

Each HLP response is plotted: in Figure 2A, the five HLPs given Sodium Thiopental, and in Figure 2B, the five HLPs given Sodium Surital. The cardiotoxicity of these drugs might be obtained.

Those animals which were maintained under artificial respiration tolerated one and one-half to three and one-half times the amount of either Surital or thiopental that was tolerated without artificial respiration.

Discussion. Based upon observations in the heart-lung preparation Surital is perhaps less toxic, but certainly no more toxic in the absolute sense, than thiopental. This is in spite of the greater anesthetic potency of Surital, requiring only about two-thirds as much in quantity to produce a similar level of anesthesia. The ability of these two thiobarbiturates to produce cardiac failure is not markedly greater than with the oxygen analogues (11). Pentobarbital produces heart failure in the identical type heart-lung preparation in dosages comparable to those described herein.

It is of major interest to know whether death of the intact animal under thiobarbiturate is respiratory (anoxic) or cardiac (direct toxicity). From the accompanying tables and description of results it can be concluded that the animal under artificial respiration is capable of tolerating much larger amounts of the thiobarbiturates than under spontaneous respiration. Also the course of death is somewhat different. With spontaneous respiration there are frequent arrhythmias, and finally there results respiratory arrest followed by a sudden drop in blood pressure and cessation of heart beat. Under artificial respiration after a long period of normal pressure, the blood pressure slowly declines until shock levels are reached and death soon ensues. Abnormal electrocardiographic changes rarely occur except as a terminal event. This slow decrease in blood pressure may be due either to a peripheral vascular collapse resulting from direct drug toxicity or paralysis of the medullary vasomotor centers. Perhaps the former is the more important.

It is apparent, therefore, that respiratory failure is the primary cause of death in the intact dog. Most, if not all, of the cardiac abnormalities are due to a progressive anoxia resulting from inadequate respiratory exchange and may be avoided by insuring adequate oxygenation of the animal tissues. It is questionable whether direct cardiac toxicity plays any role whatever in intravenous human anesthesia with the thiobarbiturates.

SUMMARY

1. In the heart-lung preparation the thiobarbiturates, thiopental and Surital, exhibit only a moderate degree of cardiac toxicity which is no greater than that produced by the corresponding oxygen analogue. On an equal dose basis, Surital is perhaps less toxic, but definitely not more toxic, than thiopental.

2. In the intact dog, anoxia secondary to respiratory failure plays the predominant role in death from thiopental or Surital anesthesia. Abnormalities of cardiac rhythm appear to take origin on the basis of inadequate oxygenation.

3. Peripheral vascular failure, of anoxic or toxic origin, may be a contributory cause of death.

REFERENCES

1. GRUBER, C. M., HAURY, V. G., AND DRAKE, M. D.: *Am. Heart. J.*, 20: 329, 1940.
2. REYNOLDS, C., AND VEAL, J. R.: *S. Med. J.*, 31: 650, 1938; REYNOLDS, C.: *Anesth. and Analg.*, 18: 270, 1939.

3. GRUBER, C. M., HAURY, V. G., AND GRUBER, C. M., JR.: THIS JOURNAL, 63: 193, 1938.
4. KOHN, R., AND LEDERER, L.: J. Lab. and Clin. Med., 23: 717, 1938.
5. BETLACH, C. J.: THIS JOURNAL, 61: 329, 1937.
6. VOLPITTO, P. P., AND MARANGONI, B. A.: J. Lab. and Clin. Med., 23: 575, 1938.
7. WOODS, L. A., WYNGAARDEN, J. B., RENNICK, B., AND SEEVERS, M. H.: Fed. Proc., 6: 387, 1947.
8. KRAYER, O., AND MENDEZ, R.: THIS JOURNAL, 74: 350, 1942.
9. WYNGAARDEN, J. B., WOODS, L. A., RIDLEY, R., AND SEEVERS, M. H.: THIS JOURNAL, preceding article.
10. WOOD, E. A., AND MOE, G. K.: Am. J. Physiol., 136: 515, 1942.
11. MOE, G. K.: Personal communication.

THE CHEMOTHERAPEUTIC AND PHARMACOLOGICAL PROPERTIES OF THE L-EPHEDRINE SALT OF PENICILLIN G (TERSAVIN¹)

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Mixtures of penicillin with vasoconstrictors such as epinephrine (Fisk and coworkers (1), Fiske *et al.* (2), Ercoli *et al.* (3) and Schachter (4)) have been used experimentally and clinically in order to obtain delayed absorption as indicated by prolonged blood levels.

It is not a rare occurrence in the field of chemotherapy and pharmacology that the characteristic effects of biologically active agents disappear if the constituents are combined in one molecule as a chemical entity even though their mixture shows the expected activity. For that reason, it appeared desirable to study the properties of an l-ephedrine salt of penicillin G which was recently prepared by Dr. M. W. Goldberg and Mr. S. Teitel in the Roche Chemical Laboratories.

The l-ephedrine salt of penicillin G (Tersavin) is a new penicillin derivative which combines crystalline penicillin G and l-ephedrine. Tersavin, which has the empirical formula: $C_{16}H_{18}O_4N_2S \cdot C_{10}H_{15}ON$, is a crystalline white powder with a melting range of 135–137°C. (decomp.). The specific optical rotation $[\alpha]_D^{20}$ in a 2.4 per cent aqueous solution is +190°. The substance is highly soluble in water (60 per cent at room temperature) and a 1.5 per cent aqueous solution has a pH of 6.2. The calculated unitage per 1 mgm. is 1187 units; this was confirmed by assay. Therefore, the potency of 1.4 mgm. of Tersavin corresponds to that of 1 mgm. of crystalline sodium penicillin G.

The present paper consists of the results of the toxicological, pharmacological and chemotherapeutic work carried out with this compound.

PART I. *Pharmacological Properties of Tersavin*

Tersavin and ephedrine HCl have been studied for their comparative toxicity, circulatory effects and bronchodilator action.

Toxicity. The toxicity values for Tersavin and ephedrine HCl were determined in mice, rats and rabbits by various routes of administration. The LD₅₀ toxicity values and their standard errors were calculated by a graphic method (5). Since the molecular weight of Tersavin (499.6) is 2.48 times that of ephedrine HCl (201.7), the data are given in terms of mgm./kgm. and millimols/kgm.; the latter shows the true relative toxicities. The data in table 1 indicate that Tersavin is only slightly more toxic than ephedrine HCl in mice and rats but about equally toxic in rabbits. The toxicity of Tersavin may, therefore, be ascribed to its ephedrine content.

¹ Tersavin—T. M.—Reg. U. S. Pat. Off.

Circulatory Effects. The relative blood pressure effects of Tersavin and ephedrine HCl were each measured in 8 dogs and 4 cats. The animals were anesthetized with dial-urethane, the drugs were administered intravenously and the blood pressure recorded with a mercury manometer. The animals were atropinized (1 mgm./kgm.) and responses to a series of graded doses of epinephrine were measured. A dose of Tersavin or ephedrine HCl was given and from the response in mm. Hg the relative potency in terms of epinephrine was estimated. A dose of Tersavin was followed by an equimolar dose of ephedrine HCl, and vice versa, in order to observe the tachyphylactic effects. Typical records from 2 dogs are shown in figure 1. This illustrates that the vasoconstrictor potencies of Tersavin and ephedrine HCl are identical on a molecular basis and each compound produces tachyphylaxis to the other. Although there is wide variation in sensitivity of animals to both Tersavin and ephedrine HCl as well as to epinephrine, the ephedrine salts are approximately 1/200 as strong as epinephrine in dogs and 1/100 as strong in cats.

TABLE 1
Toxicity of Tersavin and ephedrine HCl

SPECIES	ROUTE	EPHEDRINE HCl LD ₅₀ ± S.E.		TERSAVIN LD ₅₀ ± S.E.	
		mgm./kgm.	m mol/kgm.	mgm /kgm.	m mol/kgm.
Mice... ..	i.p.	340 ±51	1.69 ±.25	630 ±69	1.26 ±.14
Mice . . .	i.v.	95 ±19	.47 ±.09	175 ±17	.35 ±.03
Rats ..	i.p.	290 ±20	1.44 ±.10	680 ±88	1.36 ±.18
Rats	s.c.	1150	5.82	2400	4.80
Rabbits ...	i v.	65	.32	175	.35

Bronchodilator Action. The relative potency of Tersavin and ephedrine HCl as bronchodilators in comparison with epinephrine was measured on the isolated tracheal smooth muscle of guinea pigs by the method of Castillo and de Beer (6). A chain of isolated tracheal rings, attached to a light lever, was suspended in Hastings-Van Dyke solution, aerated with 95 per cent O₂: 5 per cent CO₂, and kymographic tracings of the changes in tone were recorded. The response to graded doses of epinephrine was obtained and a dose of Tersavin or ephedrine HCl was then added.

From the degree of relaxation, the potency of the ephedrine salts were estimated in terms of epinephrine. Since ephedrine salts could be washed out only with great difficulty, successive doses of the compound usually had less effect than the first so that graded responses could not be obtained. Figure 2 illustrates the bronchodilator action of Tersavin and ephedrine on different preparations. From 6 experiments with each compound an average potency of 1/500 of epinephrine was found. The effects of Tersavin were indistinguishable from those of equimolar concentrations of ephedrine HCl.

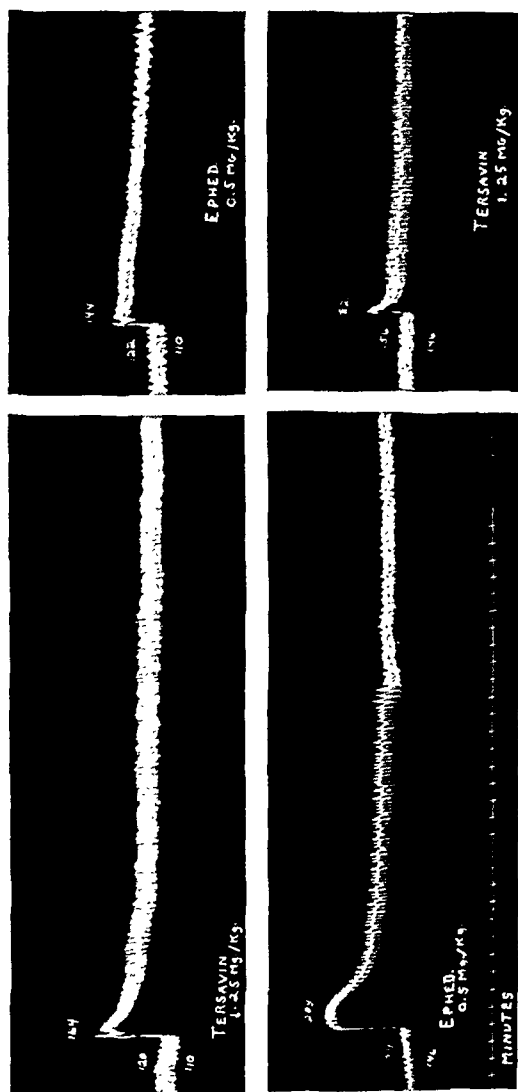


FIG. 1. CIRCULATORY EFFECTS OF TERSAVIN AND EPHEDRINE HCl

		micromoles
A. Ephinephrine.....	2 μ /kgm.	= .011
Ephinephrine.....	4 μ /kgm.	= .022
Tersavin.....	1.25 mgm./kgm.	= 2.5
B. Ephedrine HCl.....	0.5 mgm./kgm.	= 2.5
Ephinephrine.....	2 μ /kgm.	= .011
Ephinephrine.....	4 μ /kgm.	= .022
Ephedrine HCl.....	0.5 mgm./kgm.	= 2.5
Tersavin.....	1.25 mgm./kgm.	= 2.5

Time in minutes. Numbers on blood pressure records indicate mm. Hg.

PART II. *Chemotherapeutic Properties of Tersavin*

MATERIALS AND METHODS. Penicillins. The sodium salt of crystalline penicillin G (1667 units/mgm.) and the l-ephedrine salt were obtained from the Roche Chemical Laboratories.

Strains of Test Organisms. The origin of the strains used in this investigation have been described in previous publications by Schnitzer *et al.* (7). The following bacterial strains were used:

(1) *In vitro experiments*

β-hemolytic streptococci (Group A)

Strept. 4, type 3

Strept. C203, type 3

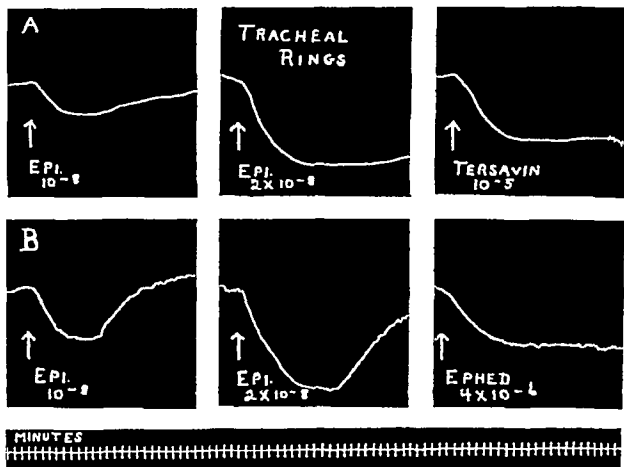


FIG. 2. ACTION ON GUINEA PIG'S TRACHEAL RINGS

- A. Epinephrine, 10^{-8} gm./cc. = 0.055 micromolar
 Epinephrine, 2×10^{-8} gm./cc. = 0.11 micromolar
 Tersavin, 10^{-5} gm./cc. = 20 micromolar
 B. Epinephrine, 10^{-8} gm./cc. = 0.055 micromolar
 Epinephrine, 2×10^{-8} gm./cc. = 0.11 micromolar
 Ephedrine HCl 4×10^{-6} gm./cc. = 20 micromolar

A and B are records from different preparations. Time in minutes.

Strept. B—from an abscess of the foot

Strept. 98—source unknown

Staphylococcus aureus

Staph. 209

Staph. L

Staph. 13—from nose culture

Staph. 49—from tonsillar culture

Pneumococci

Pn. 6301, Type I

Pn. 6302, type II

Pn. 6303, type III

Pseudomonas pyocyaneus

Strain 55—From nose culture

Strain 45—From dog's ear culture

*Organisms of the coli-typhoid group**E. coli* J*E. coli* 119—MacLeod strain*E. typhosa* I*E. typhosa* P58a*S. schottmuelleri* 10(2) *In vivo experiments* *β -hemolytic streptococci (Group A)*

Strept. 4, type 3

Strept. B

Pneumococci

Pn. 6301, type I

*Spirochetes**Borrelia* Novyi

Technique of the in vitro Tests. Serial dilutions of sodium penicillin G and Tersavin were made in the semi-synthetic medium of Adams and Roe (8) in the case of streptococci and pneumococci and in papain digest bacto-beef broth in the case of all other organisms.

The tubes were immediately inoculated with one drop of the culture dilution (streptococci—undiluted, pneumococci—undiluted; pseudomonas— 10^{-5} ; coli-typhoid group— 10^{-9}) from a 1 cc. pipette. The test tube racks were then shaken, placed in an incubator at 37°C . and read after 24 hours.

Technique of the in vivo Tests. Eighteen–twenty gram mice, taken from one breeding colony, were used in all the experiments.

(1) Systemic infections with streptococci and pneumococci. (a) Streptococci. Mice were injected intraperitoneally with 0.5 cc. of a 10^{-5} dilution of a 22-hour serum broth culture of *Streptococcus hemolyticus* #4 (Group A, type 3). This dose corresponds to 1000 MLD.

The treatment consisted of variations of our standard total dose of penicillin, 500 units/kgm. (0.3 mgm./kgm. crystalline penicillin G), as described by Soo-Hoo and Schnitzer (9). The compounds were given in 2 treatments, one immediately following infection and the other 4 hours later.

(b) Pneumococci. Mice were injected intraperitoneally with 0.3 cc. of a 22-hour serum broth culture of *Pneumococcus* 6301 (type I). This dose represents about 1000 MLD of the strain.

The treatment dose as described by Kelly and Schnitzer (10) was used. It consisted in the injection of a total of 1500 units/kgm. (0.9 mgm./kgm. crystalline penicillin G) and 3000 units/kgm. (1.8 mgm./kgm. crystalline penicillin G) in 2 treatments. The first dose was given immediately after infection while the second was given 4 hours later.

The animals in both the streptococcal and pneumococcal experiments were observed for a 3-week period. All mice that died during this time were autopsied and cultures of the heart blood were made.

(2) Systemic infection with *Borrelia* Novyi. The compounds were evaluated by the method described by Buck, Farr and Schnitzer (11). It consisted of the treatment of a fully developed borrelia infection in mice with a single standard dose of penicillin G, 25,000 units/kgm. (15.0 mgm./kgm. crystalline penicillin G). The number of spirochetes in 100 fields of the darkfield microscope was counted before the treatment and 3 and 20 hours after the treatment. From these counts, the reduction in the number of parasites in the blood was determined.

(3) Local infection. The technique of the local infection and its treatment has been previously described by us (12).

(a) Local therapeutic experiments. Two-tenths cc. of a 1:10³ dilution of a 22-hour serum broth culture of β -hemolytic streptococcus B were injected into the ventral subcutaneous tissue of mice. The animals were treated immediately, by subcutaneous injection into the infected area, with 1.0 cc. of a solution of the penicillin salts ranging from 2.5 to 20 units/cc. (0.0015-0.012 mgm./cc. crystalline penicillin G). At the end of 22-24 hours the animals were autopsied and cultures from the infected and treated areas were made on blood agar plates. Animals from which cultures showed less than 10 colonies are listed as successfully treated in the tables.

(b) Local prophylactic experiments. One cc. of a solution of the penicillin salts containing 250 or 500 units/cc. (0.15 or 0.3 mgm./cc. crystalline penicillin G) was injected into the ventral subcutaneous tissue of mice. At specified intervals of 15 minutes, 30 minutes, 1 hour and 2 hours after injection of the penicillin salts, 0.2 cc. of a 1:5 dilution of a 22-hour

TABLE 2
Comparative bacteriostatic activity of Tersavin and penicillin G in vitro

ORGANISM	BACTERIOSTATIC CONCENTRATION			
	Tersavin		Penicillin G	
	units/cc.	mgm./cc.	units/cc.	mgm./cc.
Streptococcus 4	0.0195	0.000016	0.0195	0.000012
Streptococcus C 203	0.0195	0.000016	0.0098	0.000006
Streptococcus B	0.0195	0.000016	0.0098	0.000006
Streptococcus 98	2.5	0.0021	2.5	0.0015
Staphylococcus 209	0.0195	0.000016	0.0195	0.000012
Staphylococcus L	>10.0	>0.0084	>10.0	>0.006
Staphylococcus 13	0.039	0.000033	0.039	0.000023
Staphylococcus 49	0.625	0.00053	0.625	0.00037
Pneumococcus 6301	0.0195	0.000016	0.0195	0.000012
Pneumococcus 6302	0.0195	0.000016	0.0098	0.000006
Pneumococcus 6303	0.0195	0.000016	0.0195	0.000012
Pseudomonas 35	>100.0	>0.084	>100.0	>0.06
Pseudomonas 45	>100.0	>0.084	>100.0	>0.06
E. coli J	25.0	0.021	12.5	0.0075
E. coli 119	50.0	0.042	25.0	0.015
E. typhosa F	25.0	0.021	12.5	0.0075
E. typhosa P 5S A	12.5	0.01	6.25	0.0037
S. schottmuelleri 10	50.0	0.042	25.0	0.015

serum broth culture of Streptococcus B were injected subcutaneously into the treated area. At the end of 22-24 hours, the mice were autopsied and the area of infection or infection and treatment, respectively, was swabbed. Cultures from these swabs were then made on blood agar plates.

In both types of experiments, it was unnecessary to use blood agar plates into which a penicillin-inhibitor was incorporated for making cultures from the animals, since we had found that all the penicillin had disappeared much earlier (12) and there was no danger of carrying over enough penicillin to cause any bacteriostatic activity.

* A dilution of 1:10 was used in the local therapeutic experiments, since it was found that at the time these experiments were run, a dilution 1:10 gave the same response as had a dilution of 1:5.

EXPERIMENTAL. Chemotherapeutic Activity in vitro. Eighteen strains of gram positive and gram negative bacteria were tested, by the dilution method, for their sensitivity to Tersavin and crystalline penicillin G. As table 2 shows, representative organisms of the group of penicillin sensitive and penicillin insensitive organisms were selected for these tests. One naturally resistant organism was included among the penicillin sensitive strains of staphylococci. It is evident that the bacteriostatic activity of Tersavin is identical with that of penicillin G. All differences are not greater than one dilution step and this difference is

TABLE 3

Activity of Tersavin and penicillin G in the systemic streptococcus 4 infection of mice

TOTAL DOSE	TOTAL DOSE	NO. OF MICE	SURVIVORS	CD ₅₀ ± S.E.	CD ₅₀ ± S.E.
units/kgm.	mgm./kgm.		per cent	units/kgm.	mgm./kgm.
Tersavin					
1000	0.84	29	100	220 ± 53*	0.185 ± 0.045
500	0.42	30	88.3		
250	0.21	30	58.3		
Penicillin G					
1000	0.6	30	98.4	308 ± 52	0.185 ± 0.031
500	0.3	30	85.0		
250	0.15	30	35.0		
Controls	—	30	0		

* According to the method described by De Beer (4).

TABLE 4

Activity of Tersavin and penicillin G in the type I pneumococcus infection of mice

TOTAL DOSE	TOTAL DOSE	NO. OF MICE	SURVIVORS	CD ₅₀ ± S.E.	CD ₅₀ ± S.E.
units/kgm.	mgm./kgm.		per cent	units/kgm.	mgm./kgm.
Tersavin					
3000	2.52	10	100	1300 ± 312	1.07 ± 0.26
1500	1.26	20	65		
Penicillin G					
3000	1.8	10	100	1515 ± 288	0.91 ± 0.17
1500	0.9	20	45		
Controls	—	30	0	—	—

not significant since any dilution test is only accurate to plus or minus one dilution.

Systemic Activity in vivo. (1) Anti-streptococcal activity. The results of the experiments in the streptococcal infection are given in table 3. The total dosage is based on 2 treatments given on the day of the infection at 4-hour intervals. It is evident that the effect of Tersavin is identical with that of penicillin G, the differences in the unitage of the CD₅₀ being insignificant.

(2) Anti-pneumococcal activity. The comparison of Tersavin and penicillin G in the pneumococcal infection, as given in table 4, shows that the standard

dose of 3000 units/kgm. prevented the death of all infected mice when it was given in the form of the sodium salt (1.8 mgm./kgm.) or in the form of Tersavin (2.52 mgm./kgm.). The effect of half this dose was also about the same with both drugs and the CD_{50} on the basis of unitage exhibited, therefore, no significant difference. The lower content of crystalline penicillin G made, of course, the CD_{50} by weight appear slightly better.

(3) Anti-borrelia activity. If tested by the rapid assay method with *Borrelia Novyi* (11), the activity of Tersavin was unit for unit the same as that of penicillin G (table 5). On a weight basis, about 30 per cent more Tersavin was required, which corresponds to the lower penicillin content of this salt.

If the activity of Tersavin was evaluated in a therapeutic experiment, that is by repeated treatment of a manifest blood infection, the effect was also identical with that of penicillin G. In this type of experiment, the mice received 8 subcutaneous treatments, 4 per day at 2-hour intervals, of 25,000 or 50,000 units/kgm. (15 or 30 mgm. crystalline penicillin G/kgm. or 21 or 42 mgm. Tersavin/

TABLE 5

Rapid assay of Tersavin and penicillin G in the Borrelia novyi infection of mice

Initial count: 468 ± 42 parasites/100 microscopic fields. Single subcutaneous treatment.

COMPOUND	DOSE units/kgm.	DOSE mgm./kgm.	NO. OF TESTS	REDUCTION OF PARASITES		RATIO t 3/t 20
				t 3 ^a per cent	t 20 per cent	
Tersavin.....	25,000	21.0	2	99.3	93.2	1.08
Penicillin G.....	25,000	15.0	3	99.5	92.3	1.07
Controls.....	—	—	3	65 incr. ^b	500 incr.	—

^a t 3 = count after 3 hours; t 20 = count after 20 hours.

^b incr. = % increase of spirochetal count.

kgm.) of the penicillin salts to a total of 200,000 or 400,000 units/kgm. respectively, (120 or 240 mgm. crystalline penicillin G/kgm. or 168 or 336 mgm. Tersavin/kgm.) beginning 1 day after the intraperitoneal injection of 400,000 to 500,000 parasites of *Borrelia Novyi*. The mice treated with the higher dose of the penicillin salts stayed free of relapses for a period of 3 weeks. Sixty per cent of the mice treated with the lower dose relapsed 10-14 days after the termination of therapy.

It did not seem surprising that the activity of Tersavin was not significantly different from that of penicillin G in the treatment of generalized infections. We have found in acute infections of mice that penicillin preparations showing a slow rate of absorption, induced by different vehicles or by the use of penicillin derivatives of lower solubility in water, exert the same activity as the water soluble salts of penicillin G. A similar conclusion can also be drawn from results observed by Hobby (13, 14). The anti-pneumococcal and anti-streptococcal activity of procaine penicillin of low solubility which was administered in 1 dose was about the same as that of penicillin G administered in 3 doses. It would

seem, therefore, that in the control of the acute fatal coccid infections of mice sufficient penicillin is released even if the absorption has been delayed by one means or another.

It might seem appropriate to point out that the reduction of the penicillin administration to not more than 2 treatments has been practiced in this laboratory for a long time. It is based on the early experience that in an experimental infection of mice the subdivision into small multiple doses does not offer an advantage over 1 to 2 larger doses. Similar observations have recently been published by Marshall (15), Zubrod (16), White *et al.* (17) and Gibson (18).

BLOOD LEVEL DETERMINATIONS IN RABBITS. Albino rabbits, weighing 2-3 kgm., were injected with 150,000 units (90 mgm. G; 126.4 mgm. Tersavin) dissolved in saline. Four animals were included in each group and the blood level was determined at the end of 1, 3, 5, 7 and 16 hours. At the 24-hour interval only 2 animals were used for each compound.

The bacteriostatic activity of the serum was determined in a dilution test with a β -hemolytic streptococcus (Strain #4) as the test organism. In the semi-synthetic media of Adams and Roe, used for the experiments, this strain was inhibited by 0.005-0.01 units (0.000003-0.000006 mgm.) penicillin G.

In all instances where 0.4 cc. serum or more was used in order to show growth inhibition of the organism, parallel experiments were carried out in tubes containing penicillinase. This was done in order to show that the bacteriostatic activity of the serum was actually due to penicillin.

The bacteriostatic concentrations of the serum after administration of penicillin G followed the familiar pattern. Expressed in penicillin units per cc., a peak of bacteriostatic activity was observed after 1 hour (average value: 38.4 units/cc.). This value dropped considerably after 3 hours when only 0.3 units/cc. were found. Only 1 animal out of 4 showed a value of 0.05 units/cc. after 5 hours. At later intervals no activity of the serum was observed.

The l-ephedrine salt of penicillin G (Tersavin), tested under the same conditions as above, gave similar but not identical results. The average peak concentration after 1 hour was found to be 51.2 units/cc. After 3 hours the average concentration was 18.4 units/cc., and even after 5 hours an average figure of 2.5 units/cc. was found. No demonstrable blood level was observed after 7 hours and at the later intervals.

It might be mentioned that the comparatively high value for Tersavin at the 5-hour interval was influenced by the fact that 1 rabbit exhibited a blood level of 6.4 units/cc. Even if this result were omitted from the data, the average blood level would still be 1.2 units/cc. which is considerably higher at this interval than that obtained after the administration of penicillin G.

These observations indicate that the presence of the vasoconstrictor did indeed delay the absorption of the ephedrine salt for at least 2 hours.

Differences, comparable to those observed above, may also be seen if one studies the local prophylactic anti-bacterial effect of penicillin salts. Even slight delays in absorption, such as may be induced by suspensions, have an influence which can be determined by the fate of a topical infection at the site of previous drug administration.

TOPICAL ACTIVITY IN VIVO. As described earlier in this paper, the technique

consisted in the subcutaneous infection of mice with β -hemolytic streptococci at a site where they had been treated previously with penicillin G or Tersavin. The mice were sacrificed at the end of 22-24 hours and cultures, from the treated and infected areas, were made on blood agar plates. The plates were read after 24-hours incubation.

If an interval of 15 or 30 minutes was kept between the prophylactic treatment and the infection, no significant difference was seen at the two doses in the percentage of mice successfully treated (table 6). After a 1-hour interval, differences became apparent and the number of mice in which the tissues were successfully treated was markedly higher after the administration of Tersavin than in the case of penicillin G. No significant difference was observed if 2 hours were allowed to elapse even though the percentage of successfully treated mice due to Tersavin treatment was slightly higher.

TABLE 6

Local prophylactic activity of Tersavin and penicillin G at various time intervals

COMPOUND	UNITS PER CC.	MGM. PER CC.	PER CENT MICE SUCCESSFULLY TREATED				
			15 min.*	30 min.	1 hr.	2 hrs.	Controls
Tersavin.....	500	.42	86†	82	57‡	22	0
Penicillin G.....	500	.3	73	59	23‡	0	0
Tersavin.....	250	.21	68	64	68§	22	0
Penicillin G.....	250	.15	50	64	27§	17	0

* Time of infection after treatment.

† The figures represent the results obtained with 10-30 mice for a group. Mice were considered successfully treated if the subculture on a blood agar plate showed less than 10 colonies.

‡ Significant figures because $p = 0.05$.

§ Significant figures because $p = 0.01$.

These observations seem to indicate that there is a definite tendency to prolonged prophylactic activity on the part of Tersavin.

If the technique of these experiments was changed so that the infection preceded the treatment which was administered shortly afterward, the mice were found to be successfully treated on the administration of 10-20 units penicillin G or Tersavin. This dose has been found, in many previous experiments, to be the normal dose for successful treatment of the animals. In a few instances where penicillin was more active, Tersavin was also more active.

DISCUSSION. From the work presented in the experimental parts of this paper, the conclusion may be drawn that the l-ephedrine salt of penicillin G, Tersavin, exerted the specific activity of ephedrine as well as the characteristic anti-bacterial properties of penicillin G. There does not seem to be either an antagonism or a synergism between the components since the systemic activity of l-ephedrine penicillin did not seem to be significantly increased by the vasoconstrictor. Two modifications might, however, be attributed to the presence of the vasoconstrictor. One is an influence on the blood level which is prolonged and higher

with Tersavin than in the case of penicillin G; the other is an extended prophylactic topical effect in an experimental local streptococcal infection.

We feel, therefore, that l-ephedrine penicillin, Tersavin, offers the advantages of both the antibacterial properties of penicillin G and the vasoconstrictory effect of ephedrine by the administration of a single chemically defined compound.³

SUMMARY

Experimental data are submitted which show that the l-ephedrine salt of penicillin G, Tersavin, exerts quantitatively the anti-bacterial effect of penicillin G and the vasoconstrictory properties of l-ephedrine.

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REFERENCES

1. FISK, R. T., FOORD, A. G., AND ALLES, G.: *Science*, **101**: 124, 1945.
2. FISKEN, R. A., AND GRUZHIT, O. M.: *Am. J. Syphilis*, **30**: 581, 1946.
3. ERCOLI, N., HUEPER, W. C., LANDIS, L., LEWIS, M. N., AND SCHWARTZ, B. S.: *Am. J. Med. Sci.*, **215**: 498, 1945.
4. SCHACHTER, R. J.: *Proc. Soc. Exper. Biol. and Med.*, **68**: 29, 1948.
5. DE BEER, E. J.: *THIS JOURNAL*, **85**: 1, 1945.
6. CASTILLO, J. C., AND DE BEER, E. J.: *THIS JOURNAL*, **90**: 104, 1947.
7. SCHNITZER, R. J., FOSTER, R. H. K., ERCOLI, N., SOO-HOO, G., MANGIERI, C. N., AND ROE, M. D.: *THIS JOURNAL*, **88**: 47, 1946.
8. ADAMS, M. H., AND ROE, A. S.: *J. Bact.*, **49**: 401, 1945.
9. SOO-HOO, G., AND SCHNITZER, R. J.: *Arch. Biochem.*, **5**: 99, 1944.
10. KELLY, D. R., AND SCHNITZER, R. J.: *Arch. Biochem.*, **7**: 461, 1945.
11. BUCK, M., FARR, A. C., AND SCHNITZER, R. J.: *Science*, **104**: 370, 1946.
12. GRUNBERG, E., SCHNITZER, R. J., AND UNGER, C.: *Yale J. Biol. and Med.*, **20**: 479, 1948.
13. HOBBS, G. L., BROWN, E., AND PATELSKI, R. A.: *Proc. Soc. Exper. Biol. and Med.*, **67**: 7, 1948.
14. HOBBS, G. L., LENERT, T. F., AND HYMAN, B.: *J. Bact.*, **54**: 305, 1947.
15. MARSHALL, E. K., JR.: Meeting Antibiotic Study Sec., Nat. Inst. of Health, Wash., D. C., Oct. 1, 1947.
16. ZUBROD, C. G.: *Bull. Johns Hopkins Hosp.*, **81**: 400, 1947.
17. WHITE, H. J., BAKER, M. J., AND JACKSON, E. R.: *Proc. Soc. Exper. Biol. and Med.*, **67**: 199, 1948.
18. GIBSON, C. D., JR.: *Proc. Soc. Exper. Biol. and Med.*, **67**: 278, 1948.

* Experiments which show the effect of Tersavin on the pathological and normal flora of the nasal mucosa will be reported elsewhere.

ANTAGONISTS FOR FATAL AND NON-FATAL DOSES OF QUININE INTRAVENOUSLY IN DEPRESSED CIRCULATORY STATES AND IN HYPERTHERMIA¹

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Although new anti-malarial agents are coming into widespread use, quinine will probably continue to be used, especially when intravenous use is indicated in severe falciparum malaria with coma or other cerebral symptoms (1). However, intravenous injection of the alkaloid might be hazardous, especially in pre-existing circulatory depression. Since this matter has not been previously explored, it was decided to obtain evidence on the toxicity of the alkaloid, and on the value of certain antagonists for its circulatory depression, in hemorrhage, asphyxia, hyperthermia, and shock. This paper presents the results obtained.

METHODS. The same general methods were followed as in a previous report (2). In addition to comparisons of the depressor effect of single doses (10 mgm./kgm.) of quinine in 0.9 per cent sodium chloride solution injected in 2 minutes, and the same dose of quinine mixed with an antagonistic agent, it was thought desirable that the most promising agent, epinephrine, should also be studied for possible antagonism to the fatal dose of quinine. For this purpose, the fatal dose of quinine hydrochloride was determined by continuous injection of a 1 per cent solution in normal saline solution at the rate of 2 mgm./kgm. per minute. This was then compared with the fatal dose of quinine when mixed with epinephrine in the ratio of 0.004 mgm. to 2 mgm. of quinine.

Both rabbits and cats were used. Anesthesia was produced with pentobarbital sodium, 35 mgm./kgm. being used intraperitoneally in cats, and intravenously, in rabbits. The various abnormal states were produced as follows: hemorrhagic shock by bleeding animals from the right femoral artery (blood pressures: 50-60 mm. Hg, rabbits and 70-80 mm. Hg, cats); moderate asphyxia with cyanosis and no change in blood pressure by allowing the animal to breathe through a 4-foot length of $\frac{1}{4}$ inch rubber tubing, or by clamping partially the trachea; severe asphyxia with variable though moderate blood pressure changes by using a 6-foot tube; shock with hemorrhage by opening the abdomen and gently manipulating and tugging on the mesenteries and viscera (blood pressures: 50-60 mm. Hg, rabbits and 90-100 mm. Hg, cats). For hyperthermia the body temperature of the anesthetized animals was raised to 41°-42°C. by tying the animal supine on an electrically heated pad. Although small numbers of animals were used in each state, due to non-availability, differences in effects were significant in most cases.

Small Doses of Quinine and Antagonists in Various Abnormal States (table 1). Epinephrine (0.015 mgm./kgm.) was the only drug which consistently antagonized the blood pressure lowering effect of quinine (10 mgm./kgm.) and increased recovery of blood pressure in the various abnormal states studied. After hemorrhage in rabbits, epinephrine decreased the blood pressure fall from a control of

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hemorrhage in cats. In moderate or severe asphyxia in either cats or rabbits, epinephrine was not effective as an antagonistic agent. In non-hemorrhagic shock the median fatal dose for quinine alone, in rabbits, was 90 mgm./kgm.; in cats, 71 mgm./kgm. After epinephrine was added to the quinine, the median fatal dose was 208 mgm./kgm. in rabbits, 99 mgm./kgm. in cats. In hyperthermia, the median fatal dose for quinine was 50 mgm./kgm. in rabbits, and 56 mgm./kgm. in cats. Addition of epinephrine raised the median fatal dose of quinine to 150 mgm./kgm. in rabbits, 130 mgm./kgm. in cats.

DISCUSSION. In the various abnormal states studied, namely, non-hemorrhagic shock, hemorrhage, asphyxia, and hyperthermia, the toxicity and depressor effect of quinine were almost uniformly increased. Any of these conditions might be found in clinical malaria, either as a result of the disease process itself, or of injury, or other diseases. High fever, of course, is almost always present in severe malaria, and this was found to increase the toxicity of quinine. Epinephrine, however, was quite effective in raising the fatal dose, and in antagonizing the depressor effect of quinine, thus suggesting its use in clinical malaria with high fever when quinine must be given intravenously, and assuming a similar antagonism.

Partial asphyxia in malaria might easily result from the decreased oxygen carrying capacity of the blood due to the clumping or sludging of red blood cells, or to concomitant pneumonia, or other disease process. While moderate asphyxia seemed to have little effect on the fatal dosage in animals, in severe asphyxia the toxicity of the drug was greatly increased. The lack of a beneficial effect from epinephrine suggests that clinically asphyxia or cyanosis should be treated first by oxygen inhalation before giving quinine intravenously.

Shock or hemorrhage, occurring as a result of injury or operation may accompany malaria, and both of these conditions increased the sensitivity of animals to quinine. In these conditions, epinephrine added to the quinine was effective in rabbits, but not so effective in cats.

CONCLUSIONS

1. The sensitivity of cats and rabbits to quinine was found to be greatly increased in non-hemorrhagic shock, after hemorrhage, in severe asphyxia, and in hyperthermia. In moderate asphyxia, the fatal dosage of quinine was not significantly different from that in normal animals.

2. Epinephrine given with quinine at the rate of 0.004 mgm. for each 2 mgm. of quinine was effective in increasing the fatal dosage of quinine in rabbits subjected to marked hemorrhage, non-hemorrhagic shock, and hyperthermia, but not to asphyxia. In cats, epinephrine was effective against quinine in hemorrhage and hyperthermia.

3. Of the 3 antagonists compared in rabbits, namely, epinephrine, neosynephrine, and calcium chloride, epinephrine was the only one which consistently mitigated the blood pressure lowering effect of quinine given intravenously in doses of 10 mgm./kgm. in hemorrhagic shock, non-hemorrhagic shock, hyper-

ANTAGONISTS FOR FATAL AND NON-FATAL DOSES OF QUININE INTRAVENOUSLY IN DEPRESSED CIRCULATORY STATES AND IN HYPERTHERMIA¹

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Although new anti-malarial agents are coming into widespread use, quinine will probably continue to be used, especially when intravenous use is indicated in severe falciparum malaria with coma or other cerebral symptoms (1). However, intravenous injection of the alkaloid might be hazardous, especially in pre-existing circulatory depression. Since this matter has not been previously explored, it was decided to obtain evidence on the toxicity of the alkaloid, and on the value of certain antagonists for its circulatory depression, in hemorrhage, asphyxia, hyperthermia, and shock. This paper presents the results obtained.

METHODS. The same general methods were followed as in a previous report (2). In addition to comparisons of the depressor effect of single doses (10 mgm./kgm.) of quinine in 0.9 per cent sodium chloride solution injected in 2 minutes, and the same dose of quinine mixed with an antagonistic agent, it was thought desirable that the most promising agent, epinephrine, should also be studied for possible antagonism to the fatal dose of quinine. For this purpose, the fatal dose of quinine hydrochloride was determined by continuous injection of a 1 per cent solution in normal saline solution at the rate of 2 mgm./kgm. per minute. This was then compared with the fatal dose of quinine when mixed with epinephrine in the ratio of 0.004 mgm. to 2 mgm. of quinine.

Both rabbits and cats were used. Anesthesia was produced with pentobarbital sodium, 35 mgm./kgm. being used intraperitoneally in cats, and intravenously, in rabbits. The various abnormal states were produced as follows: hemorrhagic shock by bleeding animals from the right femoral artery (blood pressures: 50-60 mm. Hg, rabbits and 70-80 mm. Hg, cats); moderate asphyxia with cyanosis and no change in blood pressure by allowing the animal to breathe through a 4-foot length of $\frac{1}{4}$ inch rubber tubing, or by clamping partially the trachea; severe asphyxia with variable though moderate blood pressure changes by using a 6-foot tube; shock with hemorrhage by opening the abdomen and gently manipulating and tugging on the mesenteries and viscera (blood pressures: 50-60 mm. Hg, rabbits and 90-100 mm. Hg, cats). For hyperthermia the body temperature of the anesthetized animals was raised to 41°-42°C. by tying the animal supine on an electrically heated pad. Although small numbers of animals were used in each state, due to non-availability, differences in effects were significant in most cases.

Small Doses of Quinine and Antagonists in Various Abnormal States (table 1). Epinephrine (0.015 mgm./kgm.) was the only drug which consistently antagonized the blood pressure lowering effect of quinine (10 mgm./kgm.) and increased recovery of blood pressure in the various abnormal states studied. After hemorrhage in rabbits, epinephrine decreased the blood pressure fall from a control of

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49 per cent for quinine alone, to 16 per cent. In non-hemorrhagic shock in rabbits; quinine produced a fall of blood pressure of 39 per cent, but with epinephrine added the fall was only 14 per cent. In partial asphyxia in rabbits, addition of epinephrine changed the blood pressure fall of quinine from 33 per cent to 15 per cent, and in hyperthermia, epinephrine decreased the quinine fall from 52 per cent to 22 per cent. Recovery of blood pressure was benefitted by

TABLE 1

Small doses of quinine and quinine with antagonists in various abnormal states

DRUGS*	RABBITS				CATS			
	No.	Trials	Aver. fall of blood pressure	Aver. recovery† of blood pressure	No.	Trials	Aver. fall of blood pressure	Aver. recovery† of blood pressure
Hemorrhage								
			<i>per cent</i>	<i>per cent</i>			<i>per cent</i>	<i>per cent</i>
Quinine.....	8	29	49 (9-73)†	86	3	11	26 (12-41)†	86
Quinine plus epinephrine.....	3	16	16 (0-29)	100	3	11	25 (0-53)	100
Non-hemorrhagic shock								
Quinine.....	9	48	39 (8-69)	89	2	9	17 (12-27)	91
Quinine plus epinephrine.....	4	27	14 (0-60)	100	2	9	8 (0-30)	95
Partial asphyxia								
Quinine.....	13	38	33 (4-69)	88	3	15	27 (15-49)	89
Quinine plus epinephrine.....	6	17	15 (0-37)	99	3	15	18 (0-33)	95
Hyperthermia								
Quinine.....	8	23	52 (21-83)	69	2	6	28 (21-35)	83
Quinine plus epinephrine.....	2	7	22 (6-38)	96	2	6	17 (6-28)	98

* Drugs (quinine 10 mgm./kgm., epinephrine 0.015 mgm./kgm.) were injected intravenously in 2 minutes.

† Range in parentheses.

‡ Compared with pre-injection level in mm. Hg.

epinephrine in each case. In cats, epinephrine was somewhat effective as an antagonist to quinine in non-hemorrhagic shock, asphyxia, and hyperthermia, but not after hemorrhage.

Neosynephrine (0.05 mgm./kgm.) and calcium chloride (10 mgm./kgm.) were also tried as antagonists to the blood pressure lowering effect of quinine, but were not significantly effective.

Fatal Doses of Quinine and Epinephrine in Normal and Abnormal States (table

The experiment illustrated in figure 3 requires further explanation. In this case a small dose of Myanesin, administered to a very rigid animal, increased the amplitude of the knee jerk and caused the appearance of clonus. This is of special interest because similar effects have been reported several times in patients. In rigid limbs both flexors and extensors are powerfully activated, and respond to the slightest stretch by reflex contraction. When a muscle contracts, its antagonist, being thereby stretched, promptly contracts also, and limits movement in the first muscle. Because of the greater importance of extensor muscles in postural activity they are considerably more spastic than the flexors. When a small dose of Myanesin is given to an animal with extreme rigidity, the spasticity in both flexors and extensors is reduced. But, whereas most of the flexor spasticity is relieved, considerable extensor spasticity remains because it was so extreme initially. Although the extensors are actually less spastic following Myanesin, they appear to be more so because they are *unopposed*. The effect of the Myanesin in this case is believed then, to be unmasking of the extensor spasticity, which had been previously limited by flexors. Additional Myanesin acts in the usual manner to reduce the remaining tone in the extensors. This differential susceptibility of extensors and flexors is probably due to fundamental differences in the pattern and amount of their innervation under the abnormal conditions of rigidity. There is no good evidence that Myanesin may actually increase spasticity in non-rigid limbs.

SUMMARY AND CONCLUSIONS

These experiments indicate that discrepancies in the results of clinical trials with Myanesin are not due to the variety of the lesions causing spasticity. Clearly, all types of experimental spasticity in cats, whatever the nature of the lesion, can be relieved by effective doses of Myanesin. The evidence presented supports the previously described theory of drug action upon facilitatory and inhibitory systems which mediate the impulses maintaining spasticity.

The authors are indebted to Miss Ethel Mary Mathis for photographing the records shown in this paper.

REFERENCES

1. BERGER, F. M., AND BRADLEY, W.: Brit. J. Pharmacol., **1**: 265, 1946.
2. HENNEMAN, E., KAPLAN, A., AND UNNA, K.: THIS JOURNAL, **97**: 331, 1949.
3. LINDSLEY, D. E., SCHREINER, L. H., AND MAGOUN, H. W.: J. Neurophysiol., **12**: 197, 1949.
4. TUREEN, L. L.: Arch. Neurol. and Psychiat., **35**: 789, 1936.
5. SNIDER, R. S., AND WOOLSEY, C. N.: Am. J. Physiol., **133**: 454, 1941.

THE SYSTEMIC ACTIONS OF THIOGLYCEROL WITH SPECIAL REFERENCE TO GOITROGENIC ACTIVITY

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While thioglycerol (TG) has been used to stimulate wound healing (1) and is used to a small extent as the active ingredient in the cold hair waving process (2), there is only limited information available on its systemic actions. Draize, Alvarez and Woodard (2) have recently reported on the chronic toxicity of percutaneously applied thioglycerol in the rabbit and on sensitization produced by this compound in the guinea pig.

In the present study, cats, rabbits, rats and mice have been used and the actions of 1-thioglycerol (TG) have been compared with those of 1,2-dithioglycerol (BAL) and thioglycolic acid (TGA). In acute experiments the actions of TG are similar to those of BAL (3-5) except that it is only $\frac{1}{4}$ to $\frac{1}{6}$ as active. Repeated administration of large doses of TG have been observed to produce mild thyroid hyperplasia in rats and rabbits.

RESULTS. Acute poisoning. Similar to BAL (3, 4) the intravenous or intraperitoneal injection of TG in toxic doses produced lacrimation, salivation, hyperexcitability, tremors, increased respiration, incoordination and convulsions (mixed clonic and tonic) in cats (i.v.), rabbits (i.v.), rats (i.p.) and mice (i.p.). Animals given fatal doses of TG usually died within six hours. Survivors of LD₅₀ doses were apparently normal 24 hours later. Application of 99.3 per cent pure TG to the clipped skin of the rabbit and rat indicates that 20 to 25 times as much TG is required to produce a systemic effect as when given by the intravenous route. Data on the toxicity of TG are given in table 1. Data obtained for the cat (4) and the rat (6) indicate that TG is $\frac{1}{3}$ and $\frac{1}{4}$ as toxic as BAL.

The effects in animals acutely poisoned with TG suggested that the mortality might be reduced by the use of a central nervous system depressant such as barbitol. The injection of barbitol ten minutes after the administration of TG did decrease the mortality. In the rat, the injection of 200 mgm./kgm. of barbitol raised the LD₅₀ of TG from 390 mgm./kgm. to 590 mgm./kgm., and 300 mgm./kgm. of barbitol raised the LD₅₀ to 700 mgm./kgm. In earlier experiments, sodium pentobarbital (30 mgm./kgm.) was found to raise the LD₅₀ by 20 per cent in cats poisoned with BAL (7).

In contrast to the signs of central nervous system stimulation seen in acute TG poisoning, TGA poisoning is characterized by a prolonged period of depression. Animals given fatal doses usually died between 12 and 36 hours after injection although the three cats given a dose of 200 mgm./kgm. died between 48 and 96 hours after injection. Data on the acute toxicity of TGA are presented in table 1. TGA possessed greater toxicity than TG in all species examined.

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Effect on circulation. TG (i.v.) in small doses (75–100 mgm./kgm.) produced a slight rise in the blood pressure of cats anesthetized with 0.5 cc./kgm. of Dial. Larger doses (>500 mgm./kgm.) produced an immediate and continuous drop

TABLE 1
Toxicity of thioglycerol and thioglycolic acid

	THIOGLYCEROL*			THIOGLYCOLIC ACID†		
	No. of animals	Conc.	LD ₅₀	No. of animals	Conc.	LD ₅₀
		%	mgm./kgm.		%	mgm./kgm.
Rat‡ (i.p.).....	48	48.3	390 ± 10§	22	53.6	165 ± 7§
Rat‡ (i.p.).....	13	6.0	400 ± 16			
Mouse (i.p.).....	26	9.7	340 ± 18	27	10.7	200 ± 13
Cat (i.v.).....	11	48.3	220 ± 10	5	53.6	ca. 175
Rabbit (i.v.).....	12	48.3	250 ± 15	7	53.6	ca. 100

* In aqueous solution.

† Administered as the ammonium salt.

‡ Sherman strain.

§ Approximate S.E. (method of Miller & Tainter).

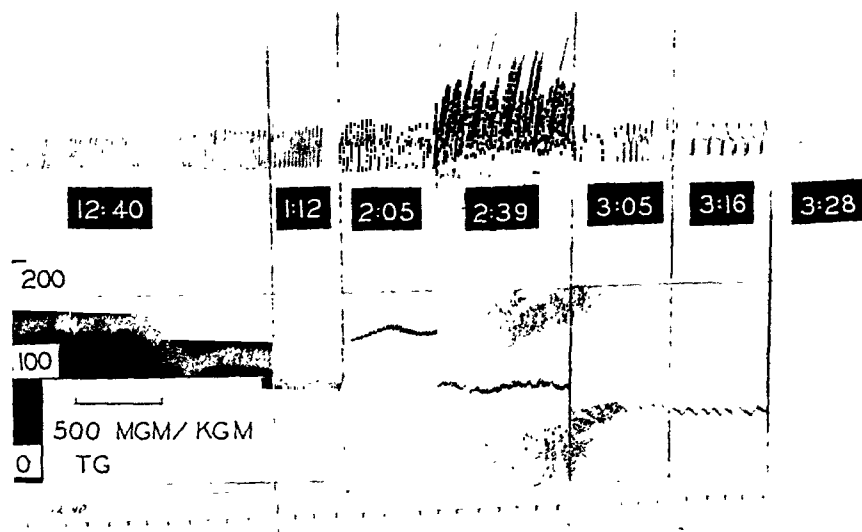


FIG. 1. THE EFFECT OF INTRAVENOUSLY ADMINISTERED TG ON THE RESPIRATION (UPPER TRACING) AND THE BLOOD PRESSURE (LOWER TRACING) OF A CAT (3.2 KG., MALE) ANESTHETIZED WITH 0.5 CC./KG. OF DIAL

The blood pressure was recorded by means of a Hürthle manometer and the respiration by the method of Pfeiffer and Moore. Timer interval—10 seconds.

to shock levels. Intermediate doses caused an immediate fall, then partial or complete recovery followed by a gradual fall to shock level. A typical experiment is shown in figure 1. The dose used, 500 mgm./kgm., is twice the LD₅₀ dose and caused the death of unanesthetized cats within one hour. In this ex-

periment, and in other similar ones, the anesthetized cats survived for two and one-half to four hours indicating that the antagonism between the barbiturates and TG previously observed in the rat also holds to some extent in the cat. In the terminal stages slight hemoconcentration (8-12 per cent) was observed and injected epinephrine produced a normal pressor response. Electrocardiograms taken throughout the course of poisoning showed no characteristic abnormalities although the heart rate was generally temporarily slowed by large doses of TG.

The data obtained on the effect of TG on the circulation are similar to those reported by Chenoweth for BAL (5), and indicate that the circulatory failure is not due to a direct action on the heart. In addition, TG in high concentrations ($4.5 \times 10^{-2} M$) did not have any measurable effect on the force of contraction of the isolated papillary muscle (8) of the cat in experiments performed by Dr. Garb.

TGA (150-200 mgm./kgm.) injected (i.v.) into cats in the form of its ammonium salt also caused a rapid fall in blood pressure to shock levels. In contrast to TG, the survival time of cats was markedly reduced in cats anesthetized with Dial.

Effect on respiration. A typical record of the effect of TG on the respiration in the cat is shown in figure 1. Following the injection (i.v.) of toxic doses of TG the respiration gradually increased in rate and amplitude. When lethal doses were administered, as in figure 1, the respiration failed after the blood pressure had fallen to shock levels.

In contrast to TG, TGA injected as the ammonium salt produced an immediate severe depression of respiration frequently lasting for from ten to fifteen minutes.

Effect on blood sugar, plasma CO₂ capacity and NPN. The i. v. injection of lethal amounts of TG (300 mgm./kgm.) into unanesthetized cats produced a rapid fall in plasma CO₂ capacity (down to 10 vol. per cent) and a rise in blood sugar (up to 350 mgm. per cent) and nonprotein nitrogen (20 per cent). Similar but smaller changes were obtained with lower toxic doses. The increase in blood sugar values was not an artefact due to circulating TG as measurements eight minutes after injection showed only a small increase. The plasma CO₂ capacity was in the normal range when measured 24, 48 and 72 hours after the injection of 200 mgm./kgm. of TG. The nonprotein nitrogen level was slightly above normal at the 48 hour point. The decreased plasma CO₂ capacity and increased blood sugar levels are similar to those observed in animals poisoned with BAL (3, 4) in which concurrent measurements of blood pH indicated that BAL produced a metabolic acidosis (3).

Cats poisoned with TGA (200 mgm./kgm.), and dying 50 to 92 hours later, showed a gradually decreasing plasma CO₂ capacity (down to 16 vol. per cent) and a gradually increasing NPN (up six fold).

Effects of repeated administration of TG. The effects of repeated administration of TG have been studied in rabbits and rats. TG (7.5 per cent aqueous solution) was applied to the clipped skin of nine rabbits six days a week for two months. Three rabbits were given a dose of 75 mgm./kgm.; three, 150 mgm./kgm.; and

three, 300 mgm./kgm. Three additional rabbits served as controls. All four groups increased in weight from approximately 1.7 kgm. to 2.6 kgm. At the conclusion of the experiment all animals were autopsied. There were no gross abnormalities with the exception of local damage (necrosis) at the site of application of TG. Microscopic examination of liver, kidney, spleen, adrenals and thymus showed no evidence of tissue damage. Thyroid sections from the rabbits receiving the highest dose showed morphologic evidence of mild hyperplasia in agreement with the report of Draize (2). As earlier work on the chronic toxicity of BAL (4) indicated that hemoglobin levels, but not the red cell count, were reduced, blood hemoglobin levels were determined on all animals on the 59th day. The average

TABLE 2
The goitrogenic action of thioglycerol in Wistar rats
(Daily percutaneous application)

EXP'T.	COMPOUND	DOSE	NO. RATS	NO. DAYS	BODY WEIGHT RANGE	THYROID WEIGHT PER 100 GM. BODY WT.	p*
		mgm./kgm.			gm.	mgm.	
1	Thioglycerol (48.3%)	500	5	23	84-114	12.6 ± 0.56	>0.01
	BAL (pure)	250	5	23	98-132	10.2 ± 0.21	0.3
	Controls	—	4	23	76-114	9.3 ± 0.75	
2	Thioglycerol (48.3%)	500	5	14	84-116	10.3 ± 0.87	0.7
	BAL (10% in oil)	250	4	14	68-126	9.8 ± 1.0	0.9
	Controls	—	5	14	88-120	9.9 ± 0.64	
3	Thioglycerol (48.3%)	500	6	27	74-134	10.0 ± .43	0.01
	Thioglycerol (48.3%)	200	4	27	112-130	9.0 ± .62	<0.1
	Controls	—	6	27	84-146	7.6 ± .65	
4	Thioglycerol (48.3%)	100	8	27	118-186	7.0 ± .35	0.7
	Controls	—	6	27	104-174	6.7 ± .58	

* Calculated by means of the Fisher t test.

value for the control rabbits was 12.9 gm./100 cc. and that in the rabbits receiving the highest dose was 13 per cent lower.

In view of the mild thyroid hyperplasia produced in the rabbit by TG applied percutaneously and the report of Graham (9) that the prolonged administration of BAL in peanut oil produced thyroid hyperplasia in the rat, the goitrogenic action of TG has been studied in greater detail in the rat. The data obtained on the goitrogenic activity of TG are summarized in tables 2, 3, and 4. Gross and microscopic examination of other tissues from the rats used in the following experiments has not disclosed any consistent tissue damage.

The data obtained when the TG was applied percutaneously six days a week are summarized in table 2. The application of TG in a dose of 500 mgm./kgm. produced a statistically significant ($p > 0.01$) increase in the thyroid weight-body weight ratio when the applications were continued over a 23 to 27 day

period but not when applied for only 14 days. Smaller doses (100 and 200 mgm./kgm.) were without effect when applied for 27 days. The percutaneous ap-

TABLE 3
The goitrogenic action of thioglycerol in rats
(Intraperitoneal injection)

EXP.	COMPOUND	DOSE	NO. INJECTIONS DAILY	NO. OF RATS	DURATION OF EXP.	STRAIN	FINAL BODY WT. RANGE	THYROID WT.-† BODY WT. RATIO	SE	†
		mgm. kgm.			days		gm.			
1	Thioglycerol (9.7% aqueous)	200	1	6	39-92	Sherman	188-294	6.3		
	Controls	—	—	2	39-92	Sherman	280-394	5.4		
2	Thioglycerol (9.7% aqueous)	300	1	3	77	Sherman	196-206	6.9		
	Controls	—	—	3	77	Sherman	270-342	7.7		
3	Thioglycerol (9.7% aqueous)	300	1	3	48	Wistar	136-150	10.3		
	Controls	—	—	4	48	Wistar	138-171	7.4		
4	Thioglycerol (9.7% aqueous)	125	2	6	14-19	Wistar	96-122	9.3	±0.19	0.3
	Controls	—	—	6	14-19	Wistar	96-120	9.9	±0.33	
5	Thioglycerol (9.7% aqueous)	100	3	7	16	Sherman	92-110	8.6	±0.66	<0.7
	Thiourea (5% aq.)	37	3	8	16	Sherman	80-106	9.7	±0.39	>0.02
	Controls	—	—	8	16	Sherman	80-106	8.1	±0.24	
6*	Thioglycerol (5% aqueous)	100	3	5	14-18	Wistar	72-86	11.6	±0.35	0.5
	BAL (5% in oil)	20	3	6	14-18	Wistar	66-112	10.6	±0.62	0.1
	Thiourea (5% aq.)	37	3	6	14-18	Wistar	76-112	14.7	±0.80	>0.01
	Controls	—	—	6	14-18	Wistar	88-102	11.9	±0.35	
7	Thioglycerol (10% in oil)	250	1	4	28	Wistar	110-208	10.0	±1.0	<0.5
	BAL (10% in oil)	50	1	5	28	Wistar	140-198	8.9	±0.20	<0.8
	Thioglycolic acid (10% in oil)	75	1	4	28	Wistar	124-208	9.2	±0.95	<0.8
	Controls	—	—	5	28	Wistar	178-190	9.3	±0.52	

* Rats in this experiment fed the purified diet.

† Mgm. thyroid weight per 100 gm. body weight.

‡ Calculated by means of the Fisher t test.

plication of BAL (250 mgm./kgm.) did not significantly increase the thyroid weight ratio at 14 or 23 days, although microscopic examination of the 23 day

TABLE 4
The goitrogenic action of thioglycerol in rats
 (Administered in diet)

EXP.†	COMPOUND	AMOUNT IN DIET	DIET	NO. OF RATS	DAYS ON DIET	BODY WEIGHT RANGE	THYROID WEIGHT* BODY WEIGHT RATIO	SE	p†
		<i>per cent</i>				<i>gm.</i>			
1	Thioglycerol	0.3	Purified	6	27-34	64-96	13.6	±1.3	>0.05
	Thioglycolic acid	0.3	Purified	6	27-34	54-104	11.5	±1.5	<0.3
	Thiourea	0.1	Purified	5	27-34	68-106	21.1	±0.49	>0.01
	Controls	—	Purified	6	27-34	80-122	9.9	±0.69	
2	Thiouracil	0.1	Dog chow	5	28	122-160	57.4	±5.4	>0.01
	Controls		Dog chow	5	28	178-190	9.3	±0.52	
3	Thioglycolic acid	0.1	Dog chow	8	28	134-170	10.1	±0.79	<0.8
	Controls		Dog chow	4	28	136-160	10.3	±0.20	

* Mgm. thyroid weight/100 gm. body weight.

† Calculated by means of the Fisher t test.

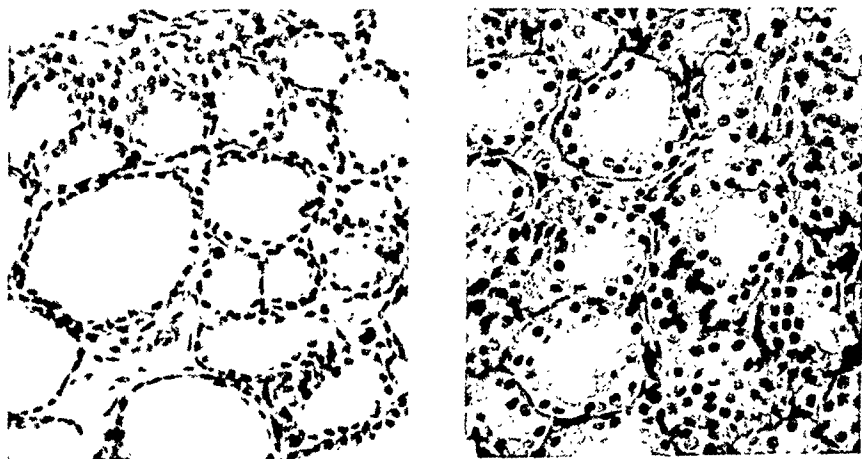


FIG. 2

A. Section from the thyroid of a control rat from experiment 1, table 2. Hematoxylin-eosin. Magnification $\times 300$.

B. Section from the thyroid of a rat from experiment 1, table 2, treated percutaneously with TG. Hematoxylin-eosin. Magnification $\times 300$.

animals disclosed an increase in the height of the epithelial cells and some evidence of colloid depletion.

The photomicrograph shown in figure 2 illustrates the thyroid changes pro-

duced by TG; they resemble those described for the sulfonamides and thiourea by the MacKenzies (10) and by Astwood (11).

The data obtained following the intraperitoneal injection of TG six days a week are summarized in table 3. No significant increase in the thyroid weight-body weight ratio was obtained with TG administered by this route although there was morphologic evidence of mild hyperplasia in the TG treated rats from experiments 1, 2, 3 and 7. In experiment 5 the injection of 100 mgm./kgm. of TG three times daily failed to produce a significant increase in the thyroid weight, whereas the injection of 37 mgm./kgm. of thiourea in the same manner did. This dosage regimen of thiourea was used as the MacKenzies (10) had found it effective in their original demonstration of the antithyroid action of thiourea. This experiment was repeated (experiment 6) with the animals maintained on a purified diet having the following composition: casein, 18 per cent; glucose, 73 per cent; corn oil, 5 per cent; salt mix (McCullum #51), 4 per cent. To each kilogram of this diet the following vitamins were added: riboflavin, 10 mgm.; thiamine, 3 mgm.; pyridoxine, 2.5 mgm.; calcium pantothenate, 7 mgm.; choline, 30 mgm.; and 5 drops of cod liver oil. This diet was prepared in an attempt to lower the iodide intake of the rats, as MacKenzie (12) had found that a low iodide diet augmented the increase in thyroid weight obtained with low levels of thiourea and thiouracil. However, although a more significant increase in thyroid weight was obtained with thiourea, a significant increase in thyroid weight was not obtained with TG.

As the failure to obtain a significant increase in thyroid weight when TG was administered intraperitoneally in aqueous solution may be due to the failure to maintain an effective concentration of TG for sufficiently long periods, TG (250 mgm./kgm.) was administered in peanut oil (experiment 7) in an attempt to secure a more prolonged action. No significant increase in thyroid weight was obtained. Similar experiments with BAL (50 mgm./kgm.) and TGA (75 mgm./kgm.) also yielded negative results.

As thiourea and thiouracil exert maximal antithyroid effects when they are incorporated in the diet, thiourea, thiouracil, TG, and TGA have been administered in this manner. The stability of TG and TGA in the mixed diets has not been determined but in an attempt to minimize losses due to their auto-oxidation these diets were prepared two to three times weekly and kept refrigerated. As expected, both thiouracil (0.1 per cent) and thiourea (0.1 per cent) produced significant increases in the thyroid weight ($p > 0.01$). TG (0.3 per cent) produced a small, but significant ($p > 0.05$), increase in thyroid weight but TGA (0.1 and 0.3 per cent) did not. In experiment 1 the purified diet described previously was used.

The results indicate that although TG possesses some goitrogenic activity it is much less active in the rat than either thiouracil or thiourea.

DISCUSSION. The toxic actions of TG, unlike those of some other compounds structurally closely related to BAL (13), resemble those of BAL with respect to signs of central nervous system stimulation and the effects on blood pressure, respiration and blood sugar and plasma CO_2 capacity. However, the degree of

hemoconcentration observed in cats injected with TG was much lower than that reported for BAL (5). Although TG temporarily slowed the heart rate, this action was not as marked as that observed with another compound related to BAL, BAL ethyl ether (13).

The actions of TGA differ from those of BAL and TG in that it has a primarily depressant action. However, TGA like BAL and TG, in toxic doses produces a severe fall in blood pressure and an acidosis. The data obtained suggest that TG and TGA, like BAL (5), produce circulatory failure because of effects on the peripheral vascular bed rather than by a direct action on the heart.

The most interesting feature of the study of the effects of repeated administration of TG, TGA and BAL was the observation that large doses of TG applied percutaneously or mixed with the diet produced a significant increase in the weight of the thyroid gland of the rat. The failure to obtain this increase when TG was administered intraperitoneally is presumably due to the failure of TG administered by this route to reach the thyroid in a sufficiently high concentration for a long enough period of time. However, even when TG was administered by this route there was morphologic evidence of mild thyroid hyperplasia. BAL in the present series of experiments produced morphologic evidence of thyroid hyperplasia but no significant increase in thyroid weight although Graham (9) found an increase in thyroid weight when BAL was repeatedly administered to rats. The failure to detect any goitrogenic action of TGA is in agreement with the results obtained in the rabbit by Draize and coworkers (2).

SH derivatives of glycerol provide the first example of simple SH compounds possessing goitrogenic activity. TGA is added to the list of inactive compounds which includes cysteine and glutathione (14).

SUMMARY

1. Acute poisoning by thioglycerol resembles that produced by BAL, but not that produced by thioglycolic acid.
2. Barbitol decreases mortality in rats poisoned with thioglycerol.
3. Thioglycerol when administered repeatedly in large doses possesses weak goitrogenic action in the rat and rabbit. Thioglycolic acid possesses no detectable goitrogenic activity.

ACKNOWLEDGEMENTS. We are indebted to Dr. E. G. McDonough of Evans Chemetics, Inc. for the samples of thioglycerol and thioglycolic acid used in these experiments, to Dr. Cosmo G. MacKenzie for helpful discussions concerning the experiments dealing with goitrogenic activity, and to the Proctor and Gamble Company for a grant which partially supported this work.

REFERENCES

1. SUTTON, L. E.: *J. A. M. A.*, 104: 2168, 1935.
2. DRAIZE, J. H., ALVAREZ, E., AND WOODARD, M.: *Fed. Proc.*, 8: 287, 1949.
3. DURLACHER, S. H., BUNTING, H., HARRISON, H. E., ORDWAY, N. K., AND ALBRINK, W. S.: *THIS JOURNAL*, 87 (Supplement): 28, 1946.

4. MODELL, W., CHENOWETH, M. B., AND KROP, S.: *THIS JOURNAL*, 87 (Supplement): 33, 1946.
5. CHENOWETH, M. B.: *THIS JOURNAL*, 87 (Supplement): 41, 1946.
6. FITZHUGH, O. G., WOODARD, G., BRAUN, H. A., LUSKY, L. M., AND CALVERY, H. O.: *THIS JOURNAL*, 87 (Supplement): 23, 1946.
7. CATTELL, McK.: *Personal Communication*.
8. CATTELL, McK., AND GOLD, H.: *THIS JOURNAL*, 62: 116, 1938.
9. GRAHAM, J. P. D.: *Biochem. J.*, 43: xxxii, 1948.
10. MACKENZIE, C. G., AND MACKENZIE, J. B.: *Endocrinol.*, 32: 185, 1943.
11. ASTWOOD, E. B., SULLIVAN, J., BISSELL, A., AND TYSLOWITZ, R.: *Endocrinol.*, 32: 210, 1943.
12. MACKENZIE, C. G.: *Endocrinol.*, 40: 137, 1947.
13. CHENOWETH, M. B., MODELL, W., AND RIKER, W. F., JR.: *THIS JOURNAL*, 87 (Supplement): 6, 1946.
14. ASTWOOD, E. B.: *The Harvey Lectures*, 195, 1944-45.

THE REMOVAL OF BROMSULPHTHALEIN FROM BLOOD PLASMA BY THE LIVER OF THE RAT^{1, 2}

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Sodium phenol tetrabromphthalein sulfonate (BSP) is one of several phthalein dyes which are eliminated almost exclusively from the blood stream by the liver. The rate of BSP removal from the blood after intravenous administration of BSP has been widely used in clinical studies of liver disease. The underlying physiological mechanisms are, however, little understood at present.

Comparison of the rate of disappearance of BSP from the blood with its excretion into the bile has shown that in the dog these two phenomena can be dissociated to some degree (1-3). Embryological studies on *Amblystoma* have demonstrated dye uptake by the liver prior to the appearance of the ability to form bile (4).

Such observations suggest that the removal of BSP from the vertebrate body can be analyzed and studied experimentally in terms of two separable phases: (1) removal from the blood stream and (2) excretion into the bile. Such an approach holds promise of at once clarifying certain fundamental phases of liver physiology, and of helping to provide a clearer evaluation and more rational application of the dye in the study of liver disease. The present paper is concerned with uptake of BSP by rat liver slices or by perfused rat livers in the absence of bile formation. It was proposed to study conditions which affect BSP uptake, to describe a mechanism which could account for the handling of BSP by the liver *in vivo*, and to furnish a basis for the study of biliary excretion of the dye.

METHODS. Recrystallized sodium phenol tetrabromphthalein sulfonate,³ $E_{1\text{cm}}^{1\%}$ (max) = 783 (pH 10, in aqueous solution, at 5787 Å) was used in all experiments. Crystalline bovine plasma albumin⁴ was used where a protein was added to the solutions.

BSP determinations were carried out by an adaptation of a method previously described (5) to a micro Evelyn colorimeter; a 10 mm. plunger cell and 5800 Å filter were used. Beer's law holds between optical densities of 0.04 and 0.5. To correct for the presence of protein the use of appropriate blank and standard determinations was found satisfactory.

Albino rats of a modified Sprague Dawley strain maintained on a diet of Purina Laboratory Chow were used. Except where stated otherwise, males weighing between 180 and 230 gm. were employed.

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³ Made available through the courtesy of Hynson, Westcott and Dunning, Inc., Baltimore, Maryland.

⁴ Supplied through the courtesy of Dr. J. B. Lesh of Armour and Company, Chicago, Illinois.

Tissue slice experiments were performed on livers obtained from rats fasted for four hours previously and killed by a blow on the head. Slices 0.5 mm. thick were cut on a Stadie Riggs microtome (6) and placed for ten minutes into a large volume of oxygenated Krebs solution (7) agitated at 35° C. The slices were transferred to Warburg flasks containing 2 cc. of media, attached to manometers and shaken at 110 strokes per minute in a constant temperature bath under 100 per cent oxygen. Changes of supernatant media were carried out outside the constant temperature bath in less than two minutes in each case.

Liver perfusions were carried out under sodium pentobarbital anesthesia through a cannula inserted into the portal vein after ligation of all tributaries cephalad to the superior mesenteric vein. Perfusion was established prior to the occlusion of the hepatic artery, an outflow being secured by the incision of the caudal end of the inferior vena cava. Experiments were rejected if a period longer than ninety seconds intervened between closure of the portal vein and establishment of perfusion flow. The perfusate was collected by means of a plastic catheter inserted into the thoracic inferior vena cava pointing caudad. A ligature tied around the vena cava below the liver closed the temporary outflow at this time. Locke's solution (8), buffered with bicarbonate was aerated with 5 per cent CO₂—95 per cent O₂ to maintain a pH of 7.4, passed through warming coils, and delivered to the vena porta at 35° C. Inflow rates were controlled by a tunnel clamp and drop counter, inflow pressure was recorded in millimeters of Locke's solution above the level of the porta hepatis by means of a manometer attached to the inflow cannula. The outflow orifice was maintained at 0 mm. of this same scale. The liver was protected by a vinylite drape except for one lobe (usually the left or medial) exposed through a slit for periodic collection of samples of transudate appearing at the surface of the liver. This was accomplished by means of tissue paper gently applied to blot off the fluid, and squeezed out into a test tube. The lobe was covered by an additional drape between collections.

In vivo studies were carried out under sodium pentobarbital anesthesia. Injections were made into the exposed femoral vein and blood samples collected by heart puncture.

Dialyses were carried out at 40° C. in Visking sausage casing bags containing 5.0 cc. of material, placed into test tubes containing 30 cc. of saline or other solution. Using a slowly rotating test tube rack a period of two days was found adequate to assure equilibrium.

RESULTS. I. Uptake of BSP by Rat Liver Slices. Rat liver slices incubated with solutions containing BSP take up considerable amounts of the dye, measured conveniently in terms of the reduction of BSP concentration in the supernatant after incubation. Unless stated otherwise, the following data are based on solutions containing 50 mgm./l. of BSP initially, incubated under the conditions outlined under e. below.

a. *Time of incubation.* Equilibrium is always reached within ten minutes at 35° C., no further change of dye distribution is observed on lengthening the period of incubation up to 60 minutes.

b. *Temperature.* Between 22 and 40° C. dye uptake is independent of temperature. At 10° C. dye uptake after 20 minutes is significantly smaller than at higher temperatures; thus, the concentration of BSP in the supernatant in a typical experiment was reduced to 22.0, 21.2, 21.0 per cent of the initial value at 22, 38, and 40° C., respectively, but only to 27.5 per cent at 10° C.

c. *pH.* Using phosphate buffers, BSP uptake is independent of pH over a range from pH 5.0 to 8.5. In ammonia buffer, too, BSP uptakes at pH 7.4 and 8.5 do not differ significantly. Using acetate buffer, however, low pH results in a markedly enhanced BSP uptake. Thus, at pH 5.0, 11.0 per cent of the initial BSP concentration remains in an acetate buffered supernatant, compared with

20.1 per cent in phosphate buffer, and 21.2 per cent and 21.9 per cent, respectively, in phosphate and acetate buffers at pH 7.4.

d. *Tissue to solution ratio.* The amount of tissue added to the incubating medium does not measurably affect the concentration of dye in the supernatant after equilibration, if the tissue amounts to more than 5 per cent of the solution. Thus, 33.2, 34.5, and 33.4 per cent of BSP remained in 2 cc. of supernatant on incubation with 0.15, 0.62 and 0.99 gm. of tissue, as against 75.3 per cent when 0.035 gm. of liver only were used.

e. *Standard conditions* for incubation experiments were selected on the basis of these findings as follows: 0.3 to 0.5 gm. tissue are incubated for 20 minute

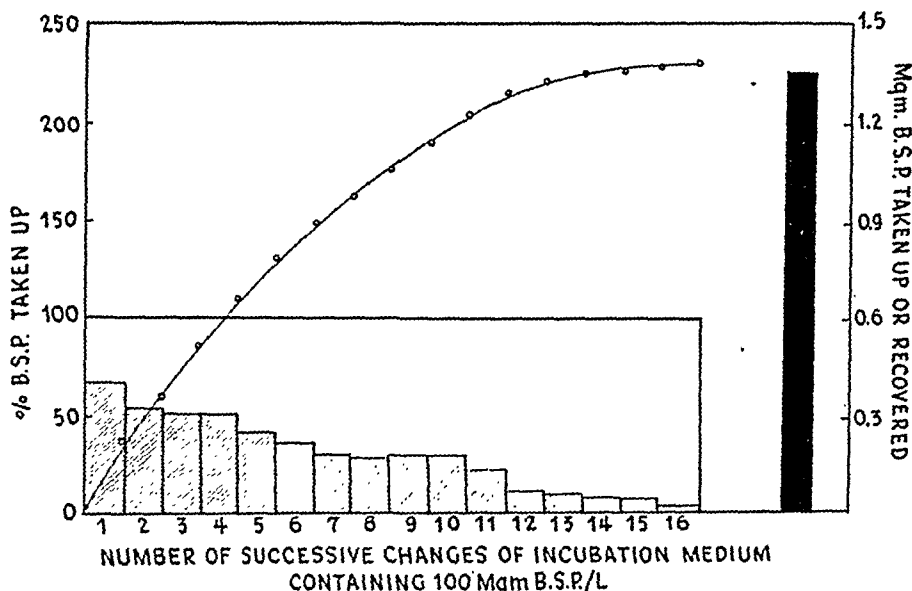


FIG. 1. BSP UPTAKE BY LIVER SLICES ON INCUBATION WITH KREBS SOLUTION CONTAINING 100 Mgm./L BSP UNDER STANDARD CONDITIONS (SEE FOOTNOTE FOR Results, I. e.).

▨ per cent BSP taken up from each solution; ○—○ cumulative amount of dye removed from supernatants; ■ dye recovered from liver slices at end of experiment.

periods in 2.0 cc. of phosphate buffered medium, pH 7.4, at 38° C. under 100 per cent CO₂.

f. *Repetitive incubations.* The tissue to solution ratio can be changed not only directly, as above, but also by transferring the slices after equilibration with one supernatant to a fresh solution, and repeating this process several times. Figure 1 shows the result of such an experiment involving sixteen transfers. The very gradual decline of BSP uptake, especially during the first four transfers, has been seen in every one of over 100 experiments of this type, and confirms the observation that the BSP concentration in the supernatant at equilibrium is but slightly dependent upon the concentration of dye within the tissue. This experiment also shows that the capacity of the tissue for BSP is limited; saturation

becomes evident after four transfers, and dye uptake almost ceases after fifteen transfers.

g. *Recovery of BSP.* Figure 1 also shows the final recovery of BSP from the incubated liver slices. In five such experiments, recoveries ranged from 90.2 to 98.5 per cent, averaging 96.3 per cent of BSP taken up. Dye recovered from liver slices cannot be separated from BSP on a chromatographic column (Al_2O_3 , H_2O —acetone— Na_2SO_4 development) (9).

h. *BSP concentration.* Slices incubated with solutions containing 5.0, 10.0, or 50.0 mgm./l. of BSP take up BSP in the same pattern: the proportion of original dye remaining in the supernatants is not detectably dependent on dye concentration within this range during the first four transfers. Thus, in a typical experiment 26.1, 25.2 and 26.2 per cent of dye remained in supernatants containing initially 5, 10, and 50 mgm./l. BSP after the first incubation, and 30.1, 36.0, 28.5 per cent after the fourth. At higher concentrations, however, saturation of the tissue becomes manifest even in the first few transfers. Thus, in the experiment quoted, incubation of slices with solutions containing 100 mgm./l. and 1000 mgm./l., respectively, 26.5 per cent and 42.3 per cent BSP remained in the first supernatants, and 41.2 per cent and 73.4 per cent in the fourth.

i. *Comparison of liver slices.* If several samples of liver slices from one rat are compared with respect to their BSP uptake, agreement of results within ± 5 per cent is generally obtained. Much wider differences are observed if slices from different animals are compared. Thus, the range of BSP remaining in the supernatants (50 mgm./l. BSP initially) was 19–29 per cent for the first and 28–47 per cent for the fourth transfer in a series of ten male animals of the same breed, and nearly the same age, weight, and dietary history.

j. *The effect of bovine plasma albumin.* The uptake of BSP by liver slices is diminished by addition of protein to the incubation medium. Figure 2 shows that at low albumin concentration the initial dye uptake decreases in proportion to the decrease of chemical potential of the dye in the protein solutions. This decrease of chemical potential (μ) results from formation of protein-dye complexes. For present purposes, μ is assumed to be proportional to the logarithm of the concentration of dialyzable dye if the final BSP concentration inside the bag is 50.0 mgm./l. At higher protein concentrations dye uptake decreases more slowly than suggested by the above relation; at 5 per cent albumin, BSP uptake of the order of 6 to 8 per cent is still observed.

As the proportion of BSP not associated with protein decreases, saturation phenomena become very prominent. At albumin concentrations above 0.2 per cent or free BSP below 5 per cent virtually no BSP is taken up after the first change of incubation solution.

Similar effects can be produced by the addition of rat serum to the incubation media—1 gm. of serum protein is roughly equivalent to 0.8 gm. of albumin in this general picture.

k. *Effect of anoxia and of various enzyme poisons.* Liver slices in an atmosphere free of oxygen (N_2) take up as much BSP as slices from the same animal incubated under O_2 . Various enzyme poisons added to the incubation media simi-

larly do not reduce the dye uptake in comparison with control slices incubated under standard conditions. The substances tested include CN^- [0.08 M (five rats) and 0.16 M NaCN (three rats) replaced an equivalent amount of NaCl in the preparation of Krebs solution; 1.0 N HCl was added to bring the mixture to pH 7.4]; Hg^{++} (0.001 M —four rats), F_2^- (0.05 M in calcium-free Krebs solution—four rats; in normal medium—three rats). These results were obtained in each case in media containing 1.0 gm./l. of bovine plasma albumin, as well as in protein-free Krebs solution.

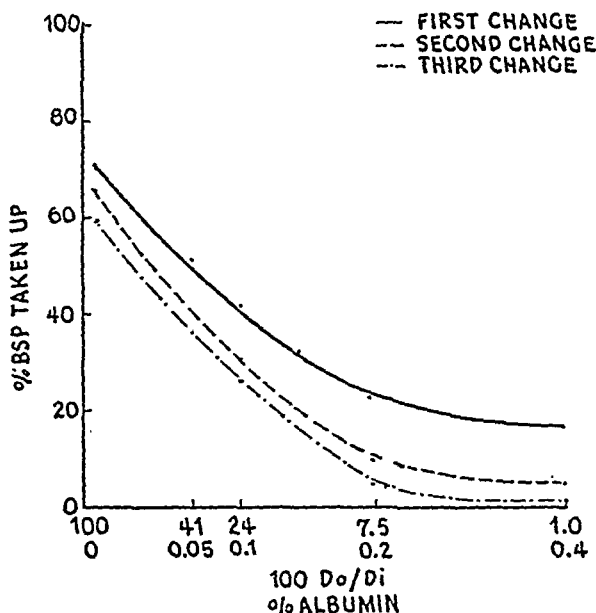


FIG. 2. BSP UPTAKE BY LIVER SLICES FROM SOLUTIONS CONTAINING 50 MG./L. AS A FUNCTION OF ALBUMIN CONTENT OR OF PERCENTAGE OF NON PROTEIN-BOUND DYE (AVERAGE OF FIVE EXPERIMENTS)

Do/Di—ratio of non protein-bound dye to total dye present in the incubation solutions.

1. *CCl₄ poisoning; India ink.* In the intact rat, the rate of removal of BSP from the blood stream is readily reduced by the administration of CCl_4 in doses that will produce an acute toxic hepatitis. Table 1 shows that under the conditions chosen, ten minutes after BSP administration the blood dye concentrations averaged nearly twice as high in the CCl_4 treated series of animals, male as well as female, as in the untreated control group. Similarly, reduced BSP clearance is observed within 30 minutes after the intravenous administration of India ink to intact rats.

In vitro, the effect of India ink can be studied by the following expedient: One liver lobe is removed after ligation from an anesthetized rat, and slices prepared and incubated as usual. India ink (0.2 cc. in 0.8 cc. 0.9 per cent NaCl) is then injected into a femoral vein and, after 30 minutes, the remainder of the

liver removed, sectioned and incubated with BSP. The dye uptakes of the original and the India ink treated lobes can be compared directly, in view of what was said above under i. In five such experiments, BSP uptake from Krebs solution without proteins as well as with addition of 0.5 and 2.0 gm./l. of bovine plasma albumin, did not show significant differences between the control lobe and the India ink treated lobes. Typical values of BSP remaining in the first supernatant are 18.2 and 18.4 per cent for control, respectively, India ink lobes in the absence of albumin, 62.5 and 59.2 per cent in solutions containing 2.0 gm./l. of albumin; values for the fourth supernatants are 30.2 per cent and 28.9 per cent, in Krebs solution, and 90.5 per cent and 91.5 per cent in 2.0 gm./l. albumin.

TABLE 1

Blood plasma BSP concentrations 10 minutes after the i.v. administration of BSP (50 mgm./kgm. body weight) to normal rats, and to India ink or CCl₄ injected rats (35 mgm./kgm. i.p. sodium pentobarbital anesthesia)

	CONTROLS†	INDIA INK‡	CONTROLS	CCl ₄ TREATED§
No. of male rats	10	10	10	9
Average weight	218.6	284.4	210.0	258.4
Plasma BSP*	28.4 ± 1.1	50.1 ± 6.2	26.1 ± 0.5	44.6 ± 5.4
± st. d. m.¶				
No. of female rats	10	10	10	10
Average weight	207.0	205.0	215.4	196.4
Plasma BSP*	36.5 ± 2.9	68.0 ± 1.9	31.0 ± 2.0	56.6 ± 4.8
± st. d. m.¶				

* 750 mgm. BSP/l. of blood plasma = 100.

† 1.0 cc. of 0.9 per cent NaCl was injected into the femoral vein 30 minutes before BSP.

‡ 0.2 cc. of Higgins India Ink was injected into the femoral vein 30 minutes before BSP in 0.8 cc. 0.9 per cent NaCl.

§ 0.5 cc. of CCl₄ per kgm. body weight was injected i.p. on each of the two days preceding the experiment.

¶ Standard Deviation of the Mean.

A direct test of this kind cannot be made in the case of CCl₄ poisoning. Statistical comparison of series of animals failed to show significant differences between normal and CCl₄ treated rats with respect to BSP uptake of slices from their livers. As an example, the ranges of BSP in the first and fourth incubation supernatants (no albumin) may be quoted: CCl₄ treated (8 rats) 14–24 per cent, mean 18.7 per cent, controls (10 rats) 19–29 per cent, mean 22.6 per cent, and CCl₄ treated 22–42 per cent, mean 31.7 per cent, controls 28–47 per cent, mean 37.6 per cent. Similar results were obtained in the presence of 0.5 and 1.0 gm./l. of albumin.

m. *Sex differences.* Table 1 shows that *in vivo* more rapid BSP clearance is obtained in male than in female rats. A sex difference could not be detected on analysis of the data for dye uptake of liver slices, nor was it observable in the perfusion experiments reported below.

n. *Uptake of BSP by tissues other than the liver; and of related phthalein dyes by liver slices.* Table 2 shows that slices from all tissues tested show some ability to take up BSP, although none quite equal the capacity for BSP uptake exhibited by liver. Comparison of various other phthalein dyes with BSP is not too readily accomplished because of the relative insolubility of many of these compounds. However, the figures shown in the second part of table 2 show that all dyes but one are taken up to a considerable extent by liver slices, and that the one that is hardly taken up by the tissue is also less firmly bound by bovine plasma albumin than the rest.

TABLE 2

The uptake of various phthalein dyes by liver slices, and of BSP by various tissue slices

	PER CENT DYE REMAINING AFTER:*			
	1	4	1	4 changes
	Krebs solution		100 mgm. albumin per Krebs solution	
50 mgm./l. BSP with:				
Diaphragm.....	29.3	50.3	53.6	80.4
Heart.....	27.7	37.7	51.7	74.8
Kidney.....	27.7	43.8	61.2	87.3
Lung.....	39.0	64.1	70.7	86.6
Spleen.....	39.2	65.3	61.5	90.2
Intestine.....	39.7	63.1	52.3	78.8
Liver.....	27.1	28.8	37.3	62.6
Liver slices with 10 mgm./l. of:			Relative binding† by bovine plasma albumin†	
BSP.....	26.1	36.8	>90	
Phenolphthalein.....	24	32	>70	
Phenoltetra bromphthalein.....	34	33	>60	
Phenolsulfonphthalein.....	81.3	85.4	<20	

* Conditions of incubation same as indicated under *Results*, I.d., except for use of different materials as shown.

†Per cent of dye non-dialyzable from solution containing 10 mgm. of dye and 100 mgm. albumin/l., pH 7.4.

II. *The Uptake of BSP by Perfused Rat Livers.* Rat livers perfused with Locke's solution remove BSP from perfusate as well as transudate.

a. *Flow rates.* Within the limits of perfusion rates set by the mechanical considerations mentioned under methods, BSP uptake remains constant for 45 to 60 minutes, and is independent of flow rate.

b. *Damage to vascular bed.* The vascular system of perfused livers may be damaged by transient excessive inflow pressures (more than 100 mm. Locke's) with histologically demonstrable vessel disruption, or by embolism. The latter condition results if either air, or India ink diluted in saline are injected directly into the perfusion cannula, or, in non-heparinized rats, if the period of portal

in these cases. At 50 gm./l. of bovine plasma albumin the early period of marked dye uptake is too short to be reliably observed. The ratio of BSP concentration in the transudate to that in perfusate at the same time are indicated to the right of table 3. The transudate-perfusate BSP ratios without exception are greater than one. The high initial values of this index decrease during perfusion, and, as saturation phenomena become marked, the ratio approaches 1.0. Preparations damaged by excessive inflow pressure, or embolism, frequently show transudate-perfusate BSP ratios below 1.0.

d. *The effect of CCl₄ poisoning, India ink, and cyanide on BSP uptake by perfused rat livers.* Livers from rats, pretreated with CCl₄ or with India ink in the same fashion as those of table 1, were perfused with protein-free or protein-containing (1.0 gm./l. albumin) solutions, 50 mgm./l. of BSP. A series of normal rats were perfused with solutions of the same BSP and albumin contents, but containing 0.15 M NaCN in place of an equivalent amount of NaCl, and brought

TABLE 4

BSP uptake by rat livers and rat hindleg perfused with solutions containing 50 mgm./l. BSP

NO. OF ANIMALS	PER CENT BSP IN PERFUSATE AFTER:			
	Minutes			
	10	20	30	45
<i>Liver</i>				
10	4.3 1.1-7.3	5.2 1.1-12	4.9 1.2-12	8.3 7.6-14
<i>Hindleg</i>				
3	38.2 33-45	51.5 45-58	62.1 58-66	84.4 75-97

to pH 7.4 with 1 N HCl. The results of these experiments, involving series of five or more animals for each type of perfusion, may be summarized as follows: In all cases, and at all time intervals used, the range of BSP concentration in transudates and perfusates, observed with the CCl₄, India ink, or CN⁻ series, fell within the normal ranges seen previously. The mean values of BSP concentrations, in all but one case, did not differ significantly from the corresponding control values. The exception was the series of CCl₄ pretreated rats perfused with 50 mgm./l. BSP—1 gm./l. albumin. Here, values of perfusate and transudate BSP during the first 20 minutes of perfusion clustered around the upper end of the normal range yielding mean values of 25.3 and 36.3 per cent of initial BSP in the perfusates at 10 and 20 minutes, and a transudate BSP of 50.5 per cent at 10 minutes. The remaining values in this case, too, do not differ significantly from the controls.

Table 4 shows a comparison between the BSP uptake of the perfused liver, and the hind legs of rats perfused through the aorta. The tissue to perfusion-

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2). This observation is in keeping with the concept advanced above, since all tissues contain intracellular proteins. *In vivo*, extrahepatic BSP uptake can be observed whenever high plasma levels of BSP are present, and result in spurious BSP clearances (12), or incomplete recoveries in the bile of the material injected (13).

While different tissues show relatively slight differences of BSP uptake on incubation, on perfusion the liver proved to be a much more effective BSP extractor than hind-limbs or heart. Thus, efficient BSP removal by the liver appears to depend on the flow of perfusate through the organ rather than on an unusual "inherent affinity" for the dye. Further light is shed on the importance for BSP uptake of the hepatic vascular organization by the following observations:

While low BSP concentrations prevail in the perfusates, transudates collected at the same time have BSP concentrations considerably higher, and in fact rather close to those observed in incubation supernatants under the same conditions (e.g., using 50 mgm./l. BSP) BSP remaining in first incubation supernatant is 22.6 per cent, in perfusate (first 10 minutes) it is 4.3 per cent, and in transudate at the same time it is 17.7 per cent; in the presence of 1.0 gm./l. of albumin the values are 56.9, 13.6 and 44.9 per cent, respectively. Disruption of the normal circulatory pattern by embolism or by transient excessive inflow pressures, was found to be the most effective way of abolishing the efficiency of BSP extraction by the perfused liver.

The results show that BSP is removed efficiently only from perfusates which follow the normal circulatory pathway. Transudate follows a random path across the liver; perfusate after disruption or blocking of its normal pathway finds alternative paths to the hepatic veins. In either case BSP concentrations remaining in the fluids are as large as (or even larger than) if tissue and solution had been equilibrated with no flow. This behavior suggests comparing the perfused liver to a multiple plate fractionating device, the successive plates of which are represented by successive portions of liver tissue, operating at every decreasing BSP concentration. Since BSP uptake by slices is proportional to the initial concentration an "equivalent number of theoretical plates" can be calculated; the perfused liver usually has an effectiveness of slightly above 3.0 plates. Such a model depends on orderly flow of the medium, and shows saturation with decreasing transudate-perfusate BSP ratios, and relative independence of flow rate and efficiency.

BSP uptake in the intact animal differs from that observed in these experiments in two major ways: (1) *In vivo* uptake from plasma continues for considerable periods of time, while the liver perfused with solutions of comparable protein content is "saturated" in a few minutes; and, (2) Several agents which affect BSP uptake *in vivo* fail to do so *in vitro*. These points will be discussed in detail elsewhere (13). Continuous BSP uptake by the liver can be obtained only if biliary excretion of the dye functions normally. Thus, while coupling of BSP uptake and of the energy requiring excretion of the dye is loose, it is detectable under conditions of extended BSP supply to the liver. The second point appears

to be linked to the fact that *in vivo* agents such as CCl_4 or India ink induce very marked demonstrable circulatory changes (14, 15), which for various reasons (e.g., absence of innervation and vasomotion in the perfused livers, perhaps lack of erythrocytes in medium) are not observable in the perfused organ.

With these limitations dye uptake by the perfused liver during the early period preceding saturation amounts to about 15 per cent from 5 per cent albumin. Direct data for the intact rat are not yet available; in the dog the concentration of BSP in hepatic venous blood is 15 to 40 per cent [see e.g. (11)] lower than in the portal venous blood. In the rat slightly lower values would be expected on the basis of the rates of dye clearance. These figures indicate that the dye uptake observed *in vitro* is of a magnitude comparable with that observed *in vivo*.

The unique position of the liver in respect to BSP elimination from the blood stream, in view of the findings reported here, appears not to be based on a special affinity for the dye by the tissue as a whole, or special cells within it. Rather the circulatory arrangement allowing for the multiple plate effect discussed above, and the ability of the liver to "desaturate" itself by reexcreting into bile taken up, render the liver a much more effective BSP extractor than any other organ.

SUMMARY

1. Rat livers can take up bromsulphthalein (BSP), either on perfusion or on incubation of tissue with solutions containing BSP.

2. BSP uptake proceeds unabated in the presence of CN^- , F^- , Hg^{++} ions, and hence does not appear to involve metabolic reactions.

3. Dye taken up by the tissue can be recovered to the extent of 90 to 98 per cent in a form indistinguishable from BSP.

4. Other tissues are capable of taking up BSP on incubation nearly as actively as liver slices. However, the liver is much more effective than hindlimbs or heart in removing BSP from solutions perfused through the organ.

5. In the perfused liver, very efficient extraction of BSP is observed only from perfusate that has followed the normal circulatory path; transudate, or perfusate collected after damaging the vascular system of the liver by embolism or excessive inflow pressures have BSP concentrations that are as high as those of incubation supernatants.

6. CCl_4 poisoning or India ink injections, while reducing BSP clearance *in vivo*, do not significantly affect BSP uptake by liver slices or by perfused livers.

7. In the presence of bovine plasma albumin, BSP uptake *in vitro* is reduced in comparison with protein-free media. Saturation of dye uptake also is observed sooner under these conditions. Livers perfused with 5 per cent albumin solutions, will remove about 15 per cent of BSP from the perfusion solution during the first ten minute period.

8. Uptake by liver slices of other phthalein dyes has been studied. Phenol-sulfonphthalein is the only one of these poorly taken up; also, it alone is but slightly bound by bovine plasma albumin.

9. The data have been discussed from the points of view of the mechanism of dye uptake, the structural factors underlying the efficient BSP uptake by the

perfused liver, and the relation of the dye uptake *in vitro* to phenomena in the intact animal.

It is a pleasure to acknowledge the able assistance of Mr. Nicholas Nicosia who carried out many of the perfusion experiments, and of Miss Neysa Phares who assisted with some of the analyses.

REFERENCES

1. CANTAROW, A., AND WIRTS, C. W.: Proc. Soc. Exper. Biol. and Med., **47**: 252, 1941.
2. WIRTS, C. W., AND CANTAROW, A.: Am. J. Digestive Diseases, **9**: 101, 1942.
3. WIRTS, C. W., AND CANTAROW, A.: Am. J. Digestive Diseases, **10**: 261, 1943.
4. ARMSTRONG, P. B.: Fed. Proc., **6**: 70, 1947.
5. BRAUER, R. W., AND ROOT, M. A.: Am. J. Physiol., **149**: 611, 1947.
6. STADIE, W. C., AND RIGGS, B. C.: J. Biol. Chem., **154**: 687, 1944.
7. UMBREIT, W. W., BURRIS, R. H., AND SHAFFER, J. F.: Manometric Techniques, p. 194, 1945.
8. SOLLMAN, T. H., AND HANZLIK, P. J.: Fundamentals of Experimental Pharmacology, 2nd ed., p. 95, 1939.
9. KREBS, J. S., AND BRAUER, R. W.: Fed. Proc., **8**: 310, 1949.
10. CLAUDE, A.: J. Exper. Med., **84**: 51, 1946.
11. BRAUER, R. W., AND PESSOTTI, R. L.: Fed. Proc., **7**: 207, 1948.
12. COHN, C., LEVINE, R., AND KOLONSEY, M.: Am. J. Physiol. **155**: 286, 1948.
13. BRAUER, R. W., KREBS, J. S., AND HIGGINS, L.: to be published.
14. HIMSWORTH, H. P.: Lowell Institute Lectures, 1947.
15. BRAUER, R. W., AND NOTHACKER, W.: Fed. Proc., **8**: 364, 1949.

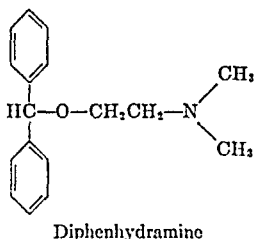
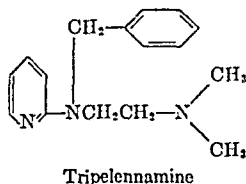
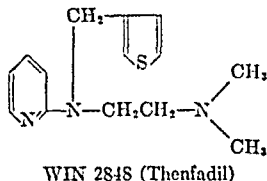
THE TOXICOLOGIC PROPERTIES OF N,N-DIMETHYL-N'-(3-THENYL)-N'-(2-PYRIDYL)ETHYLENEDIAMINE HYDROCHLORIDE (THENFADIL): A NEW ANTIHISTAMINIC DRUG

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In general, the active antihistaminic compounds fall into two main classes: ethers and amines. Diphenhydramine is a representative of the first, whereas tripeleminamine is a representative of the second class. N,N-dimethyl-N'-(3-thenyl)-N'-(2-pyridyl)ethylenediamine (WIN 2848)¹ has two heterocyclic substituents. It differs from tripeleminamine in that a 3-thenyl group is substituted for the benzyl group:



The pharmacology and antihistaminic properties of WIN 2848 have been described by Lands *et al.* (1). Luduena and Ananenkov (2) found this compound to be quite effective in relieving the characteristic histamine skin reactions when both drugs were applied to the intact human skin. The toxicity of antihistaminic drugs in experimental animals is characterized mainly by violent excitement and convulsions followed by prostration (3-11). In contrast to this, the clinical manifestations of toxicity usually include drowsiness, lassitude, headache, nausea and dizziness (12-14). While the toxicity symptoms in animals do not appear to resemble those observed in man, they do emphasize the need for caution in the

¹ WIN 2848 has been given the trade name 'Thenfadil' by Winthrop-Stearns, Inc.

clinical use of these drugs, particularly where dosages may tend to approach those known to produce distinct symptoms in animals. The present communication describes the toxicologic properties of WIN 2848 ('Thenfadir') with particular reference to comparative acute toxicity studies on tripeleennamine and diphenhydramine.

METHODS. WIN 2848, tripeleennamine and diphenhydramine were used in the form of the hydrochloride salts either in aqueous solution or as the solid crystalline material.

Acute toxicity. The test animals were male albino Webster strain mice (22 ± 2 gm.), Sherman strain male albino rats (100 ± 10 gm.), Syrian hamsters (100 ± 10 gm.), rabbits of mixed albino and chinchilla stock (2.5 ± 0.5 kgm.) and mongrel dogs (6-10 kgm.). The volumes of solution employed were 0.35 \pm 0.15 cc. for the small animals, 1.0 cc./kgm. for the rabbit and 0.1 to 1.0 cc./kgm. for the dog. The rate of intravenous injection was 1.0 cc./minute. The animals were housed in a well lighted and ventilated room maintained at 24.5° C., with a nutritionally adequate diet and with water available at all times. The $LD_{50} \pm$ s.e. was estimated by the method of Miller and Tainter (15) at 24 hours after medication with continued observations made for a period of seven days for any delayed toxic manifestations.

Subacute toxicity. WIN 2848 was administered subcutaneously once daily, 6 days a week for 3 weeks to groups of 5 rats each at doses of 5, 10, 20 and 40 mgm./kgm. The doses were made up in distilled water and administered in a constant volume of 0.5 cc./100 gm. of body weight. The control group was given distilled water. The rats were weighed three times weekly and the total volume of injection adjusted accordingly after each weighing.

Chronic toxicity. Two groups of three healthy mongrel dogs were given doses of 5 and 10 mgm./kgm. (except as noted later in the text) of WIN 2848 orally by capsule once daily six days a week over a period of 90 days. The dose for each dog was mixed with sufficient lactose to make a total of 300 mgm. in each capsule. A third group of three dogs received 300 mgm. of lactose over the same period as a control. The dogs were weighed once a week and adjustments made in the total daily dosage according to the body weight. Hematologic examinations were made at weekly intervals. Blood chemistry studies, including blood glucose, non-protein nitrogen, blood chloride, albumin, globulin and total plasma protein estimations were made at 1, 2, 4, 8 and 12 week intervals. At the conclusion of the test the dogs were sacrificed for gross and histopathologic examination.

RESULTS. *Acute toxicity.* In general, the pattern of events following medication was the same for all three drugs in each of the species studied, regardless of the route of administration. After intravenous injection of WIN 2848, extreme hyperexcitability, thrashing about the cage, vocalization, localized skeletal muscle tremors rapidly developing into violent tonic clonic spasms and opisthotonos became apparent within one to five minutes. The onset of symptoms occurred in five to ten minutes after intramuscular, subcutaneous and intraperitoneal injection and in ten to twenty minutes following oral administration. The animals which recovered from sublethal doses lay panting and prostrate from exhaustion for 30 to 60 minutes after cessation of the convulsive seizures. Increased muscle tonus was observed in all species throughout most of the recovery period. The dose-mortality curves were very steep in all cases. Death invariably occurred during or immediately following one of the convulsive seizures with respiratory failure preceding cardiac arrest. No delayed deaths were observed after 24 hours.

As seen in table 1, the average acute toxicity of WIN 2848 appears to be approximately 10 per cent greater than that of tripeleennamine by intravenous injection in four species, and 30 to 100 per cent greater by subcutaneous and intra-

peritoneal injection in mice. By oral administration to mice, the acute toxicity of WIN 2848 appears to be approximately 30 per cent greater than that for tripeleppamine but about 30 per cent less toxic than diphenhydramine.

TABLE 1

Comparison of the acute toxicity of WIN 2848 with tripeleppamine and diphenhydramine expressed in mgm./kgm. as the hydrochloride salt

SPECIES	ROUTE OF ADMINISTRATION	WIN 2848		TRIPLEPPAMINE		DIPHENHYDRAMINE		RELATIVE TOXICITY TRIPELEPPAMINE = 1	
		No. of animals	LD ₅₀ ± s.e.	No. of animals	LD ₅₀ ± s.e.	No. of animals	LD ₅₀ ± s.e.	WIN 2848	Diphenhydramine
Mouse	Intravenous	40	14.2 ± 1	30	17 ± 1.4	30	35 ± 1	1.2	0.5
	Subcutaneous	30	36 ± 4	40	73 ± 6	40	144 ± 8	2.0	0.5
	Intraperitoneal	30	55 ± 5	60	70 ± 2	60	80 ± 8	1.3	0.9
	Oral	30	277 ± 15	30	360 ± 30	30	200 ± 25	1.3	1.8
Rat	Intravenous	30	15 ± 1	30	13 ± 1		—	0.9	—
	Oral	30	525 ± 50*						
Hamster	Intravenous	30	9 ± 1	30	13 ± 1	9	18	1.4	0.7
Rabbit	Intravenous	30	12 ± 1	9	11		—	0.9	—
Dog	Intravenous	8	10						
	Intramuscular	7	12						
	Oral	5	60						

* Sherman strain, male albino rats weighing 160 ± 15 gm.

TABLE 2

Subacute subcutaneous toxicity of WIN 2848 in rats

DAILY DOSE	TOTAL ADMIN.	MORTALITY		MEAN BODY WEIGHT		GAIN IN	
		At 24 hours	At end of test	Initial	Final	Grams	Per cent
mgm./kgm.	mgm./kgm.			grams	grams		
5	90	0/5	0/5	135.0	200.8	65.8	49
10	180	0/5	0/5	138.0	193.6	55.6	40
20	360	0/5	1/5	142.0	202.8	60.8	43
40	720	0/5	5/5	142.0	—	—	—
Control....	—	0/5	0/5	134.4	202.0	67.6	50

Subacute toxicity. The mortality and mean body weight data are shown in table 2. No significant differences were found between the mean body weight of the control group and that of the groups at 5, 10 and 20 mgm./kgm. No deaths occurred at dosages of 5 and 10 mgm./kgm. One rat died after nineteen days at 20 mgm./kgm. and all five rats were dead by the twentieth day at 40 mgm./

kgm. Symptoms of intoxication were absent at 5 mgm./kgm. No symptoms were observed at the 10 mgm./kgm. dose level until the seventeenth day when three of the five rats became hyperexcitable and experienced brief tonoclonic spasms. At 20 mgm./kgm. a mildly hyperexcitable state obtained during the first week with convulsive seizures appearing during the second week and continuing throughout the remainder of the test period. Within five to ten minutes after the first injection of 40 mgm./kgm., the rats became extremely excited and belligerent, slashing viciously at each other. Opisthotonos with tonic and clonic spasms of three to four minutes' duration occurred at irregular intervals for one to two hours daily after medication. In spite of the severity of the reactions, no deaths resulted until the ninth day of the test. The deaths usually occurred at the termination of one of the convulsive seizures and were associated with

TABLE 3
Chronic oral toxicity of WIN 2848 in dogs

DAILY DOSE	DOG NO.	SEX	BODY WEIGHT		GAIN IN BODY WEIGHT	
			Initial	Final	Kgm.	Per cent
mgm./kgm.			kgm.	kgm.		
5	48-4944	F	10.4	11.0	0.6	5.8
	48-4949	M	10.0	11.9	1.9	19.0
	48-4928	F	17.8	18.6	0.8	4.5
10	47-3553	M	11.7	12.1	0.4	3.4
	48-4932	M	10.6	12.0	1.4	13.2
	48-4946	M	13.2	13.8	0.6	4.6
Control	48-4940	F	12.2	13.5	1.3	10.7
	48-4931	M	12.3	14.0	1.7	13.8
	48-4937	F	9.7	12.2	2.5	25.8

respiratory arrest. No evidence of hematologic changes were found. Gross examination at autopsy and subsequent histopathologic studies revealed no evidence of pathologic changes resulting from the medication with WIN 2848.

Chronic toxicity. All of the dogs survived the 90-day chronic toxicity test. As shown in table 3, each of the dogs gained weight. The average percentage increase in body weight at the end of the test was 10 per cent for the group at 5 mgm./kgm. and 7 per cent for those at 10 mgm./kgm. compared with a 17 per cent increase for the control group. No significant hematologic changes were found. The blood glucose, non-protein nitrogen, blood chloride, albumin, globulin and total plasma protein values remained relatively unchanged throughout the test. There were no pathologic tissue changes noted which might be attributed to the medication.

The group of dogs at 5 mgm./kgm. remained symptom-free throughout the test. Some evidence of hyperexcitability consisting of restlessness with barking and whining became apparent in two of the three dogs at 10 mgm./kgm. after

the third week. These symptoms were obscure at times and did not increase in intensity with continued medication. The third dog of this group did not show any symptoms of intoxication at 10 mgm./kgm. No distinct evidence of drowsiness was observed. The dose levels were raised from 5 and 10 mgm./kgm. to 10 and 20 mgm./kgm., respectively, for five days during the tenth week of medication in an effort to establish a maximum tolerated dose. The dogs which had been receiving 5 mgm./kgm. promptly began showing distinct symptoms of intoxication characterized mainly by a mild hyperexcitability with occasional brief convulsive seizures. The group which had been receiving 10 mgm./kgm. became quite excitable and apprehensive at 20 mgm./kgm., and all three dogs experienced one or more brief but severe epileptiform convulsive seizures during this five-day period. The doses were returned to their previous levels of 5 and 10 mgm./kgm. at the end of the tenth week for the remaining three weeks of the test. The severe symptoms of toxicity disappeared as soon as the doses were reduced. There were no symptoms of toxicity at 5 mgm./kgm. and the behavior of each of the dogs at 10 mgm./kgm. returned to the same pattern which had obtained before increasing the doses.

Pharmacodynamic studies. The intravenous effects of graduated doses of WIN 2848 were studied in four pentobarbitalized dogs. The first indication of systemic toxicity appeared as a depressor effect on the blood pressure which became greater as the dose was increased. Doses of 2 to 8 mgm./kgm. produced a fall of 20 to 50 mm. of Hg with a return to pre-injection level in approximately ten minutes. Doses in this range were without significant effect on the pulse or respiratory rates. At doses of 16 to 25 mgm./kgm., the blood pressure fell precipitously accompanied by an increase in respiratory rate and with variable effects on the pulse rate. One dog died within five minutes following an initial dose of 20 mgm./kgm. while the other three dogs survived total doses of 48, 50 and 60 mgm./kgm.

Potentiation of Evipal-induced sleep. In view of the demonstration of the potentiating effect of antihistaminic drugs on barbiturate-induced sleep in mice by Winter (16), it was deemed advisable to investigate the sedative effect of WIN 2848 in a similar manner. Groups of 30 mice each were injected with 100 mgm./kgm. of Evipal intraperitoneally and the waking time for each mouse recorded according to the criteria described by Winter. Three additional groups of 30 mice each were pretreated subcutaneously with 10 mgm./kgm. of WIN 2848, tripeleennamine and diphenhydramine 30 minutes before intraperitoneal injection of 100 mgm./kgm. with Evipal. The mean waking time in minutes \pm its standard error was calculated for each of the four groups. The results are shown in table 4.

It was found that diphenhydramine significantly prolonged the waking time of the mice while WIN 2848 and tripeleennamine had no significant effect.

Discussion. The comparative acute intravenous toxicity data indicate that the inherent toxicity of WIN 2848 is of the same order of magnitude as that of tripeleennamine. Both compounds, however, are more toxic than diphenhydramine. By subcutaneous administration to mice, WIN 2848 appears to be twice

kgm. Symptoms of intoxication were absent at 5 mgm./kgm. No symptoms were observed at the 10 mgm./kgm. dose level until the seventeenth day when three of the five rats became hyperexcitable and experienced brief tonic-clonic spasms. At 20 mgm./kgm. a mildly hyperexcitable state obtained during the first week with convulsive seizures appearing during the second week and continuing throughout the remainder of the test period. Within five to ten minutes after the first injection of 40 mgm./kgm., the rats became extremely excited and belligerent, slashing viciously at each other. Opisthotonos with tonic and clonic spasms of three to four minutes' duration occurred at irregular intervals for one to two hours daily after medication. In spite of the severity of the reactions, no deaths resulted until the ninth day of the test. The deaths usually occurred at the termination of one of the convulsive seizures and were associated with

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SUMMARY

1. The acute intravenous toxicity of WIN 2848 was found to be similar to that of tripeleonnamine in mice, rats and rabbits, but significantly greater in hamsters. Both compounds were more toxic than diphenhydramine by intravenous, subcutaneous and intraperitoneal injection in mice. The acute oral toxicity of WIN 2848 in mice was similar to that of tripeleonnamine and diphenhydramine.

2. When administered subcutaneously to rats once daily, six days a week for three weeks, WIN 2848 caused no deaths at 5 and 10 mgm./kgm., one death at 20 mgm./kgm. after nineteen days of medication and killed all of the test animals by the twentieth day at 40 mgm./kgm. No significant effect on body weight was encountered at doses of 5, 10 and 20 mgm./kgm. No significant hematologic or pathologic changes were found.

3. WIN 2848 administered orally to dogs in doses of 5 and 10 mgm./kgm. once daily, six days a week for 90 days resulted in no deaths or loss in body weight. No significant changes were observed in hematology, blood glucose, non-protein nitrogen, blood chloride, albumin, globulin or total protein values. No tissue changes were found which could be attributed to the drug.

4. No signs of intoxication were observed upon repeated administration of 5 mgm./kgm. either subcutaneously in rats for three weeks or orally in dogs for three months. Evidence of cumulative toxicity was observed at doses of 10 and 20 mgm./kgm. upon subcutaneous administration in rats.

5. No evidence of cumulative toxicity was observed with oral medication in dogs at doses of 5 and 10 mgm./kgm.

6. The signs of acute intoxication with WIN 2848 were, in general, similar to those of known, commercially available antihistaminic drugs.

7. The mean waking time of mice from Evipal-induced sleep was prolonged 8 per cent by WIN 2848, 11 per cent by tripeleonnamine and 43 per cent by diphenhydramine.

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REFERENCES

1. LANDS, A. M., HOPPE, J. O., SIEGMUND, O. H., AND LUDUENA, F. P.: *THIS JOURNAL*, 95: 45, 1949.
2. LUDUENA, F. P., AND ANANENKO, E.: *In press*.
3. MAYER, R. L., HAYS, H. W., BROUSSEAU, D., MATHIESON, D., RENNICK, B., AND YONKMAN, F. F.: *J. Lab. Clin. Med.*, 31: 749, 1946.
4. GRUNZIT, O. M., AND FISKEN, R. A.: *THIS JOURNAL*, 89: 227, 1947.
5. ROTII, L. W., RICHARDS, R. K., EVERETT, G. M., AND SHEPPERD, I. M.: *Arch. internat. de pharmacodyn. et de Therap.*, 75: 362, 1948.

6. ERCOLI, N., SCHACHTER, R. J., HUEPER, W. C., AND LEWIS, M. N.: THIS JOURNAL, 93: 210, 1948.
7. LITCHFIELD, J. T., JR., ADAMS, M. R., GODDARD, L., JAEGER, M. S., AND ALONSO, L.: Bull. Johns Hopkins Hosp., 81: 55, 1947.
8. LEHMAN, G.: THIS JOURNAL, 92: 249, 1948.
9. BROWN, B. B., AND WERNER, H. W.: J. Lab. Clin. Med., 33: 325, 1948.
10. REINHARD, J. F., AND SCUDI, J. V.: Proc. Soc. Exper. Biol. and Med., 66: 512, 1947.
11. WINTER, C. A.: THIS JOURNAL, 90: 224, 1947.
12. FEINBERG, S. M.: J. A. M. A., 132: 702, 1946.
13. BROWN, S. F.: Proc. Soc. Exper. Biol. and Med., 67: 373, 1948.
14. HALEY, T. J.: J. Am. Pharm. Assoc., 37: 383, 1948.
15. MILLER, L. C., AND TAINTER, M. L.: Proc. Soc. Exper. Biol. and Med., 57: 261, 1944.
16. WINTER, C. A.: THIS JOURNAL, 94: 7, 1948.

COMPARISON OF SMOOTH MUSCLE EFFECTS OF CROTONOSIDE (ISOGUANOSINE) AND ADENOSINE

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Emil Fischer (1) in 1897 reported the synthesis of a number of purine derivatives. Among these was isoguanine or 2-oxy-6-amino purine. He considered this compound as an oxidation product of adenine and predicted that it would be found in the animal organism or perhaps might already have been isolated but mistaken for guanine. In 1932 a glycoside was isolated from the croton seed by Chetbuliez and Bernard (2), called "crotonoside" and identified as 2-oxy-6-amino purine riboside or isoguanosine. The aglycone proved to be identical with Fischer's synthetic product. Even earlier (1927), Buell and Perkins (3) reported the isolation of oxyadenine (2-oxy-6-amino purine) from pig's blood, thus revealing the occurrence of this substance in the animal organism. The closely related nucleoside, adenosine (6-amino purine riboside), has been extensively studied and shown to be an integral part of essential enzymes (4) as well as having characteristic pharmacological effects (5-8). Little however is known about the physiological or pharmacological properties of crotonoside. Parnas and Ostern (9) in a study of adenine derivatives on the isolated perfused heart mentioned that crotonoside has activities similar to adenosine although weaker and more prolonged. We have found that crotonoside is considerably more active than adenosine as tested on the isolated uterus and intestine of the guinea pig, rabbit and hamster, and on the blood pressure of the rabbit and cat.

METHODS *Blood pressure.* The carotid blood pressure of anesthetized rabbits was recorded by a Hg manometer and that of anesthetized cats by both a Hg manometer and a Harvard membrane manometer simultaneously. Pentobarbital sodium was used as the anesthetic, 25 mgm. per kgm. of body weight intravenously for the rabbits and 30 mgm. per kgm. of body weight intraperitoneally for the cats. The drugs were injected into a marginal ear vein of the rabbits and into a femoral vein of the cats.

Isolated smooth muscle. Activity of sections of ileum from rabbits, guinea pigs and hamsters and of uterine horns from guinea pigs and hamsters (killed by a blow on the head) were recorded, using the Magnus technique with a 50 cc. U.S.P. Locke-Ringer bath at 37.5° C. and aerated with oxygen. The volume of the drug solutions added did not exceed 0.2 cc.

Drugs. The adenosine used in this study was prepared from yeast and verified for purity by one of the authors (F. S.). The isoguanosine (crotonoside) was kindly supplied by Dr. J. R. Spies of the Allergen Division of the U. S. Department of Agriculture. Solutions of the drugs were made in physiological saline to a concentration of not over 0.005 M.

RESULTS. Intravenous injection of crotonoside in the anesthetized rabbit caused a prompt decrease in blood pressure with slow recovery to the normal level. Equivalent reduction of blood pressure levels was obtained with adenosine

with approximately three times the dose required for crotonoside, 0.4 micromols of crotonoside equaling 1.25 micromols of adenosine. The duration of adenosine action was considerably shorter than that of crotonoside. No tachyphylactic action has been noted.

In the anesthetized cat, the carotid blood pressure was reduced to a much greater degree (~ 44 mm. Hg) by a dose of 0.5 micromols per kgm. body weight

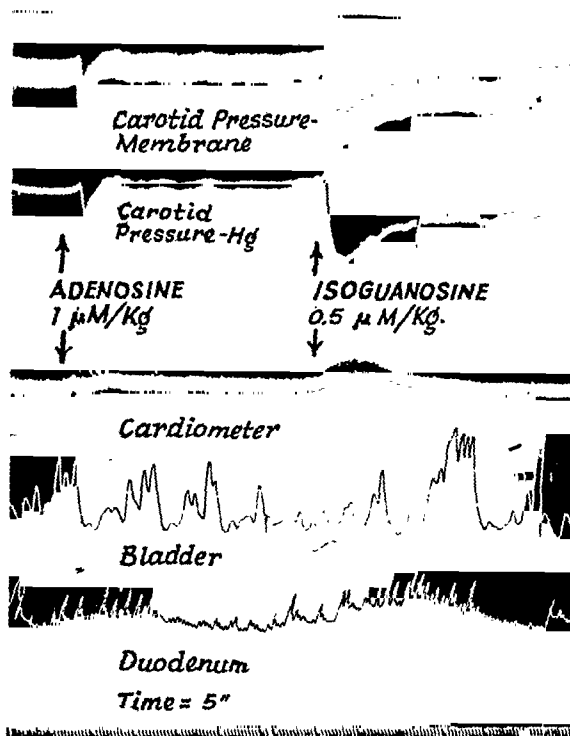


FIG. 1. EFFECT OF DRUGS AFTER INTRAVENOUS INJECTION IN AN ANESTHETIZED CAT, UNDER ARTIFICIAL RESPIRATION

Cardiometer tracings were made by the method of Gregory and Ewing (Tex. Reports on Biol. and Med., 1: 153, 1943); bladder and intestine tracings by balloon-tambour system.

of crotonoside than by a dose of 1 micromol per kgm. body weight of adenosine (~ 16 mm. Hg) (figure 1), indicating a ratio of at least 5:1 in relative activity.

The amplitude of the contractions of isolated rabbit intestine was depressed by both adenosine and crotonoside, the latter drug again being the more active and of greater duration (figure 2). A bath concentration of 2×10^{-5} *M* adenosine caused a decrease in amplitude approximately equal to a concentration of 8×10^{-6} *M* crotonoside, indicating a ratio of activity of about 1:2½. No effect on rate of rhythmic contractions was evident.

The isolated guinea pig uterus responded to these drugs by an increase in tone without much change in the minor rhythmic contractions originally present

in some preparations. The experiment illustrated in figure 3 shows that the activity of a $2 \times 10^{-5} M$ concentration of adenosine was between that of a 2×10^{-7} and $3 \times 10^{-7} M$ concentration of crotonoside, or that the latter drug had a

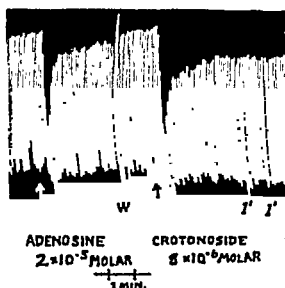


FIG. 2. ISOLATED STRIP OF INTESTINAL MUSCLE OF A RABBIT (MAGNUS PREPARATION)

Drug dosage expressed as final bath concentration. W = wash with fresh Locke-Ringer sol. and drum stopped 5 minutes. Drum stopped for 2 one-minute intervals as indicated by I'.

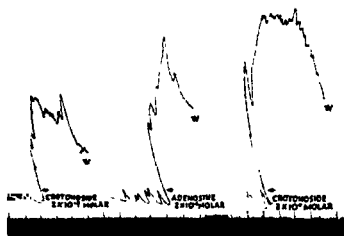


FIG. 3. ISOLATED UTERINE HORN OF GUINEA PIG

Drug dosage expressed as final bath concentration: W = wash with fresh Locke-Ringer sol. and drum stopped 5 minutes. Time: 5-second intervals with breaks at 1 minute.

60-100 fold greater activity. Other guinea pig uterine strips varied in this respect but were always twenty or more times as sensitive to crotonoside as to adenosine.

The tone of isolated guinea pig intestine was decreased by both drugs, 0.05 micromol of crotonoside being as active as 1 micromol of adenosine or twenty times more effective (figure 4).

Isolated tissues of the hamster reacted qualitatively to adenosine and crotonoside similar to guinea pig tissues, but the difference in effectiveness of the

two compounds was less marked and approximated the relative effectiveness as seen on the blood pressure and isolated intestine of the rabbit. Thus a 4×10^{-6} *M* concentration of crotonoside was approximately equal to a 1×10^{-5} *M* concentration of adenosine in inhibiting the intestinal tone and in stimulating both tone and rhythmic contractions of the uterus, or a ratio of $2\frac{1}{2}:1$.

DISCUSSION. The frequently found phenomenon of species variation in response to drugs is encountered in this study. Guinea pig tissues responded in a manner to indicate a more than twenty-fold greater activity of crotonoside over adenosine, whereas experiments with rabbits, cats and hamsters showed a

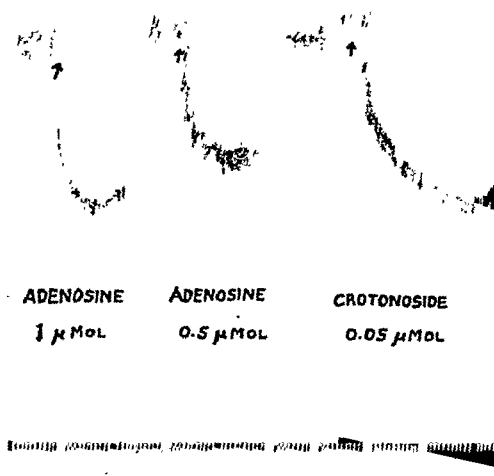


FIG. 4. ISOLATED INTESTINAL STRIP OF GUINEA PIG

Amount of drug as indicated added to the 50 cc. Locke-Ringer bath at arrows. Five-minute recovery intervals. Time: 5-second intervals with breaks at 1 minute.

two and one-half to five-fold difference. This emphasizes the need of caution in translating results obtained in one species to probable effects in another.

The observation that crotonoside is considerably more active than adenosine on the smooth muscle here reported was somewhat unexpected since crotonoside (isoguanosine) may be considered as 2-oxy-adenosine or an oxidation product of adenosine as suggested by Fischer (1). Frequently, oxidation products are less active biologically and may be assumed to be the result of detoxifying processes. Here, however, the oxy-compound is definitely more active and thus corresponds for example to the increased effectiveness of oxophenarsine over its parent substance, arsphenamine.

The related purine compounds examined by others, such as the adenylic acids, adenosine phosphates, cozymase, etc. were suspected to act by one mechanism, i.e., being interconverted either to adenosine or adenosine-5-phosphate and then deaminated. This common denominator seems to be out of the question

for crotonoside since it is much more active than adenosine or its phosphate. An interference with some enzyme system of muscular contraction may provide a better explanation. Perhaps most adenine compounds are degraded to adenosine upon injection and the latter interferes with ATP action in Szent-Györgyi's actomyosin (10, 11). Crotonoside may be a better inhibitor than adenosine and the animal organism may not be able to convert crotonoside to adenosine. It has been shown previously that adenosine deaminase does not attack crotonoside (12) nor does purine nucleosidase split the glycoside (unpublished results). Thus its fate in the mammalian organism is obscure. The greater activity and more prolonged effects of crotonoside as compared to adenosine may be related to the lack of ability of these enzymes to inactivate or destroy this glycoside, thereby allowing the maintenance of its full effects for a longer period of time.

Among various decomposition products of nucleoproteins, Torda and Wolff (13) found that adenosine and inosinic acid inhibit the synthesis of acetylcholine by minced frog brain and infer that this may be an explanation for the activity of such products. Crotonoside was not tested for this effect so that we have at present no evidence concerning the effect of the latter drug on acetylcholine synthesis.

Related purine, pyrimidine and triazine derivatives are being studied in this laboratory in an attempt to find common factors in the chemical structure which may be related to pharmacological activity. Parnas and Ostern (9) pointed out the importance of the 6-amino group of adenosine for its cardiac effects on the frog. There are indications that substitutions on other parts of the ring also affect the pharmacological activity in a rather specific manner.

SUMMARY

Crotonoside (isoguanosine) has been shown to be much more active than adenosine in reducing the blood pressure in rabbits and cats, in decreasing the tone of isolated intestinal strips of the rabbit, guinea pig and hamster and in stimulating the isolated uterus of guinea pigs and hamsters.

The difference in activity of these two compounds is considerably more marked with the guinea pig tissues than with those of the rabbit, cat or hamster.

Possible relationships to theories of action of adenosine are discussed.

REFERENCES

1. FISCHER, E.: *Ber.*, 30: 2226, 1897.
2. CHERBULIEZ, E., AND BERNARD, K.: *Helv. Chim. Acta*, 15: 464, 1932.
3. BUELL, M. V., AND PERKINS, M. E.: *J. Biol. Chem.*, 72: 745, 1927.
4. KALCKAR, H. M.: *Ann. Rev. Biochem.*, 14: 283, 1945.
5. VON EULER, H., VON EULER, U. S., AND SCHLENK, F.: *Acta Physiol. Scand.*, 1: 19, 1940.
6. BENNET, D. W., AND DRURY, A. N.: *J. Physiol.*, 72: 288, 1931.
7. FLOSSNER, O.: *Arch. Exper. Path. u. Pharmacol.*, 174: 245, 1933.
8. DEUTICKE, H. S.: *Arch. f. d. ges. Physiol.*, 230: 537, 1932.
9. PARNAS, J. K., AND OSTERN, P.: *Klin. Wchnschr.*, 11: 1551, 1932.
10. SZENT-GYÖRGYI, A.: *Chemistry of muscle metabolism*. Academic Press, N. Y., 1947.
11. SZENT-GYÖRGYI, A.: *Nature of life*. Academic Press, N. Y., 1948.
12. SCHAEDEL, M. L., WALDVOGEL, M. J., AND SCHLENK, F.: *J. Biol. Chem.*, 171: 135, 1947.
13. TORDA, C., AND WOLFF, H. G.: *Proc. Soc. Exper. Biol. & Med.*, 58: 108, 1945.

CARDIAC EFFECTS OF METHOXAMINE (β -[2,5-DIMETHOXY-PHENYL]- β -HYDROXYISOPROPYL AMINE HCl) AND DESOXYEPHEDRINE DURING CYCLOPROPANE ANESTHESIA¹

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The choice of a pressor agent for use during cyclopropane anesthesia has been shown to be critical. Primary and secondary sympathomimetic amines with a catechol nucleus may cause ventricular tachycardia or ventricular fibrillation. Amines with no hydroxyl groups and those with an hydroxyl group in the 4-position produce supraventricular tachycardia (1, 2). In view of the favorable clinical report concerning desoxyephedrine (methedrine) as a pressor agent (3) it was decided to determine the effects of this compound and another promising amine, methoxamine, (β -[2,5-dimethoxyphenyl]- β -hydroxyisopropyl amine HCl) on cardiac rhythm during cyclopropane anesthesia.

METHODS. The same procedure was followed as in previous investigations of 26 sympathomimetic amines (1, 2). Twenty-five mongrel dogs of both sexes weighing from 7 to 16 kgm. were used in this study. The amines were employed in amounts which gave a pressor response equivalent to the standard dose of epinephrine of 0.01 mgm./kgm. Pressor ratios were determined in the conventional manner by cannulating a femoral artery and recording mean arterial blood pressure with a mercury manometer. Only one drug was compared with epinephrine in each animal in one day.

In order to obtain a better comparison of the cardiac effects of the pressor agents the same animals were used for each amine. However, at least two days were allowed to elapse between the injection of methoxamine and desoxyephedrine. Electrocardiograms were also taken during the injection of the amines into the same unanesthetized animals. In all experiments the stylus of the electrocardiograph was under constant observation until the blood pressure returned to normal. Records were taken at short intervals and with any change of rhythm.

RESULTS. In table 1 are summarized the results of the blood pressure studies. One mgm./kgm. of methoxamine is seen to cause a pressor effect comparable to the standard dose of epinephrine of 0.01 mgm./kgm. The equivalent dose of desoxyephedrine is 2.0 mgm./kgm. More than 60 minutes were usually required for the blood pressure to return to pre-injection levels.

The effects of the three amines on cardiac rhythm are summarized in table 2. Methoxamine does not result in disturbances of rhythm in the heart which is sensitized by cyclopropane. None of the 25 animals had tachycardia. This includes the five animals which were given one and a half times the equipressor dose.

¹ Supported in part by a grant from Burroughs Wellcome and Company, Inc.

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TABLE 1

Pressor responses to methoxamine, desoxyephedrine and epinephrine in dogs anesthetized with cyclopropane

(Maximum rise in mean arterial blood pressure in mm. Hg)

DOG NO.	EPINEPHRINE	METHOXAMINE
	0.01 mgm./kgm.	1.0 mgm./kgm.
1	80	68
2	92	98
3	66	48
4	44	42
5	70	86
6	76	98
7	70	98
		DESOXYEPHEDRINE
		2.0 mgm./kgm.
8	69	61
9	64	62
10	66	48
11	63	64
12	87	85

TABLE 2

Comparison of effects on cardiac rhythm of methoxamine, desoxyephedrine and epinephrine

	DOSAGE MG./KG.	NUMBER OF DOGS	S-A BRADYCARDIA (<60 /min.)	S-A TACHYCARDIA (>225 /min.)	A-V BLOCK	A-V PREMATURE CONTRACTIONS	A-V BRADYCARDIA (<60 /min.)	A-V TACHYCARDIA (>225 /min.)	FEW VENTRICULAR* PREMATURE CONTRAC.	MANY VENTRICULAR† PREMATURE CONTRAC.	VENTRICULAR RHYTHM (<170 /min.)	VENTRICULAR TACHY- CARDIA (>225 /min.)	VENTRICULAR FIBRILLATION
Methoxamine (unanesthetized).....	1.0	20	18		8		9		4		7		
Methoxamine (with cyclopropane).....	1.0	20	3		1						1		
Methoxamine (unanesthetized)‡.....	1.5	5	5		4				1		1		
Methoxamine (with cyclopropane)‡.....	1.5	5					1		1		2		
Epinephrine (unanesthetized).....	0.01	25	12	1	4	1	5		7	5	4		
Epinephrine (with cyclopropane).....	0.01	25						6		1		25	3
Desoxyephedrine (unanesthetized).....	2.0	21	6	2		2			4	2	7	3	
Desoxyephedrine (with cyclopropane).....	2.0	21		20				4	8	2			

* Few ventricular premature contractions = less than 1 premature contraction to 6 supraventricular beats.

† Many ventricular premature contractions = more than 1 premature contraction to 6 supraventricular beats.

‡ One and one half times the equipressor dose.

Note: Since certain animals had more than one type of arrhythmia, the total incidence in certain cases is greater than the number of animals used.

Desoxyephedrine gives no evidence of ventricular stimulation but does increase supraventricular activity during cyclopropane. Twenty of the 21 animals had sinoauricular tachycardia at rates in excess of 225 per minute.

Epinephrine resulted in ventricular tachycardia in all 25 animals. In three this serious arrhythmia progressed within a few seconds to fatal ventricular fibrillation.

DISCUSSION. Cyclopropane has been shown to increase the irritability of the heart reflexly (4, 5). Primary and secondary aromatic amines with no hydroxyl groups on the ring or with an hydroxyl in the 4-position have a preferential stimulating effect on supraventricular regions and thus cause sinoauricular tachycardia in the cyclopropane-sensitized heart. Desoxyephedrine belongs to this class. The results are similar to those reported previously with ephedrine, amphetamine, Paredrine and phenylpropanolamine.

Primary and secondary aromatic amines with a catechol nucleus preferentially stimulate below the A-V node in the heart sensitized by cyclopropane and consequently cause ventricular tachycardia and ventricular fibrillation. Such agents as epinephrine, arterenol, Cobefrine, Epinine, and Kephine are absolutely contraindicated during cyclopropane anesthesia.

Methoxamine, with methoxy groups in the 2- and 5-positions, gives no evidence of increase in irritability of the cyclopropane-sensitized heart. Phenylephrine was the only one of 26 primary and secondary aromatic amines studied previously which was found equally innocuous during cyclopropane anesthesia.

SUMMARY

Methoxamine (β -[2,5-dimethoxyphenyl]- β -hydroxyisopropyl amine HCl) does not increase the irritability of the cyclopropane-sensitized heart. It is a safe pressor agent for use during cyclopropane anesthesia.

Desoxyephedrine causes sinoauricular tachycardia in the cyclopropane-sensitized heart.

Methoxamine and desoxyephedrine (methedrine) used in this study were supplied by Dr. D. S. Searle, Medical Director, Burroughs Wellcome and Co., Inc., Tuckahoe, New York.

Epinephrine (Adrenalin HCl) was kindly furnished by Parke Davis and Company.

REFERENCES

1. ORTH, O. S., LEIGH, M. D., MELLISH, C. H., AND STUTZMAN, J. W.: *THIS JOURNAL*, 67: 1, 1939.
2. ORTH, O. S., STUTZMAN, J. W., AND MEEK, W. J.: *THIS JOURNAL*, 81: 197, 1914.
3. DRIFTS, R. D., AND DEMING, M. V. N.: *Surg. Gynec. and Obstet.*, 83: 312, 1916.
4. ALLEN, C. R., STUTZMAN, J. W., AND MEEK, W. J.: *Anesthesiol.*, 1: 158, 1910.
5. STUTZMAN, J. W., MURPHY, Q., ALLEN, C. R., AND MEEK, W. J.: *Anesthesiol.*, 8: 579, 1947.

BIOLOGICAL ASSAY OF ANTIHISTAMINICS, ATROPINE AND ANTISPASMODICS UPON THE GUINEA PIG GUT

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The method described in a previous paper (1) for estimating antagonism potency has been developed to study several questions dealing with the mechanism of inhibition produced by antihistaminics, atropine and antispasmodics. The main characteristic of the method consists in using time of recovery as a measure of the intensity of the effect produced by the inhibitor. The R_{50} index was defined as the time in seconds necessary for a 50 per cent recovery after washing out of the inhibitor. Another characteristic of the method is to employ all the responses of the muscle to a constant standard dose of the active agent (histamine or acetylcholine) as data for the estimation of the potency of the antagonist employed. For each response, a constant k' was calculated and the average k' for each concentration of the inhibitor constitutes a separate measure of its potency. These two quantities R_{50} and k' were found to be experimentally and theoretically related through a simple equation that is graphically represented by the equilateral hyperbole. The present paper deals with some developments of the method and especially with its application to problems connected with the mechanism of action of this class of drugs.

EXPERIMENTAL. All experiments were performed according to the directions listed in our previous paper (1). In the assays in which high concentrations of cations were used, the muscle was left for a long time in contact with the new Tyrode solution until normal contractility was observed. Since all data used in the present paper are calculated as percentages, it was always found possible to increase or decrease the concentration of the spasmogenic agent, in order to get comparable results when the perfusing bath was switched to a new Tyrode solution with a different salt concentration. Concentrations as high as five times the KCl or $MgCl_2$ or $CaCl_2$ of the standard Tyrode could be applied without any damage to the contractile mechanism of the gut, provided a sufficient interval of time was allowed to elapse in order to permit the muscle to establish equilibrium with the new environment. With $SrCl_2$, moderate concentrations produced an irreversible spasm of the muscle and henceforth our trials with that cation were limited to a concentration of 0.0075 M added to the standard Tyrode solution. With $BaCl_2$ no workable concentration could be found to obtain useful information, since it produced a sort of hyperexcitability even when added at very low concentrations.

The initial responses of the muscle were taken as 100 per cent and the percentage response after one minute (time 0) after washing out of the inhibitor was taken as P_0 and P was the observed percentage response after time t . Constant k' was calculated according to the formula:

$$k' = \frac{100}{tP} \log \frac{100 - P_0}{100 - P} \quad (1)$$

The index R_{40} (found) was read directly from the curves of recovery, as the abscissa (in seconds) of the intersection with the 50 per cent ordinate. The constant R_{40} (calc.) was calculated from k' by the equation:

$$R_{40} = 36.1/k' \quad (II)$$

The antihistaminics used were the same as in the previous report (1), plus the Italian Mg.322 (2-imidazolinylmethyl-benzhydryl ether) introduced by Cavalini (2).

RESULTS. *Assays with different antagonists toward histamine.* Accuracy in the results obtained with the method described above depends largely upon variation arising from three sources. First, the constant k' is an average of several values each calculated for a separate response of the muscle to the spasmogen so that it reflects the *momentaneous* variability of the biological structure employed. Variability of k' also depends upon the order of magnitude of k' itself. In fact, it has been verified that the SE (standard error) for larger values of k' is larger than should be expected from the ratio of its value to the average. The higher variability of k' when the recovery is too fast is due in part to the exponential nature of the phenomenon. Furthermore, when k' is large, R_{40} is small, this indicating that recovery is complete in a short interval and, therefore, only a few data are available for the calculation of k' .

The repetition of the assay after recovery of the gut has been completed introduces a second source of variation. This variation will limit the sensitivity of the method. In general, it has been possible to discriminate between 2 and 5 microgm. of diphenhydramine (Benadryl), but hardly so between 1 and 2 microgm. If one has to compare different antagonists, it is necessary to choose a range of concentrations giving discriminative responses. The usual procedure of alternating additions of the antagonists to be compared, has also been found to constitute a favorable condition. The third source of variation derives from changing the piece of gut. This variation is so large that no useful data could be obtained upon different pieces of intestine. In common with all biological assay methods of the kind, it is necessary here to use a reference standard drug. Benadryl was chosen as the standard (potency 1) because it occupies an intermediate position in the series of drugs tested.

As shown in table 1, increasing doses of the antihistaminics produce decreasing values for the constant k' . From several data of that kind, we could conclude that Mg.322 is less potent than Benadryl and probably more so than Antistin. Taking Benadryl as standard (potency 1), the potency of the other antihistaminics can be calculated, as indicated in table 2.

Antagonism between atropine and acetylcholine. One of the advantages of the method described is its applicability to different pairs of antagonist-spasmogenic drugs, upon the same intestinal muscle, at the same time. If one alternates histamine and acetylcholine as the testing agent, interesting "spectra" of inhibition can be obtained as described before (1). This fact would suggest that the same mechanism is involved in the recovery after inhibition produced by antagonists toward histamine and those toward acetylcholine. The analogy can be

pushed forward by applying the formulas for the constant k' and R_{50} to the antagonism atropine vs. acetylcholine. The striking result obtained indicates that the same mathematical relationships hold when atropine is used as the antagonist and acetylcholine as the testing drug.

TABLE 1

Comparison between Benadryl, Antistín and Mg. 322 upon the same guinea pig gut

ANTAGONIST	DOSE ADDED	D. F.	$k' \pm SE$	R_{50} (FOUND)	R_{50} (CALC.)
				<i>sec.</i>	<i>sec.</i>
Antistín	2.0	2	0.25 ± 0.026	96	130
	5.0	2	0.14 ± 0.021	360	280
	10.0	7	0.07 ± 0.007	630	515
Mg. 322	2.0	4	0.10 ± 0.006	216	360
	5.0	4	0.09 ± 0.005	396	400
	5.0	5	0.09 ± 0.004	450	400
	10.0	7	0.06 ± 0.006	756	580
Benadryl	2.0	5	0.09 ± 0.009	423	400
	2.0	7	0.08 ± 0.007	510	450
	5.0	9	0.05 ± 0.004	750	710

Legend: d.f. means degrees of freedom in the calculation of SE; R_{50} (found) was determined directly upon the recovery plots; R_{50} (calc.) was calculated from equation II and k' represents the average of all values obtained by applying equation I to all experimental data (percentages of recovery) for each dose of the antagonist.

TABLE 2

Relative potency of several antihistaminics and antispasmodics as inhibitors of the histamine effect upon the guinea pig ileum

ANTAGONIST	RELATIVE POTENCY
Neo-antergan.....	>20
Pyribenzamine.....	10
Benadryl.....	1
Mg. 322.....	0.4
Antistín.....	0.33
Trasentine.....	0.007
Pethidine.....	0.002

Legend: The data presented in this table are approximate estimates from many different assays in which pairs of drugs have been assayed upon the same piece of gut, taking always Benadryl as the running standard.

As shown in fig. 1, if the intensity of inhibition is measured in terms of R_{50} (time in seconds for a 50 per cent recovery) a linear relationship between log-dose and R_{50} values is also verified, as shown before for lysocithin and antihistaminics. Table 3 indicates the numerical values for constants k' and R_{50} obtained when increasing doses of atropine were used and velocity of recovery

tested with the same standard dose of acetylcholine (usually 0.1 or 0.2 cc. of a 1:10 million solution).

A simple comparison between data obtained with the two classes of antagonists shows clearly that the order of magnitude and the kind of connection between k' and R_{50} are almost the same for the antagonism antihistaminics vs. histamine and atropine vs. acetylcholine. This analogy becomes even more striking if we note as indicated in the next paragraph that the points determined by the two

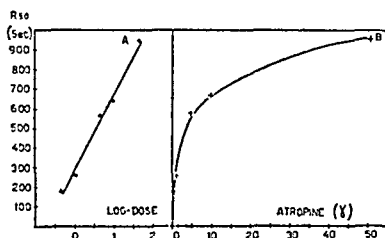


FIG. 1. ANTAGONISM ATROPINE-ACETYLCHOLINE

The values for R_{50} (found) for increasing doses of atropine are plotted: (A) Against the logarithms of the dose of atropine and (B) against the dose in arithmetic scale.

TABLE 3

Values for constant k' and index R_{50} obtained with increasing doses of atropine

DOSE OF ATROPINE	$k' \pm SE$	R_{50} (FOUND)	R_{50} (CALC.)
microgm.		seconds	seconds
0.5	0.270 ± 0.027	125	102
1.0	0.176 ± 0.015	212	144
5.0	0.056 ± 0.010	649	672
10.0	0.049 ± 0.004	740	780
20.0	0.034 ± 0.001	1062	1176
50.0	0.029 ± 0.0001	1250	1380
100.0	0.023 ± 0.001	1570	1512

constants in the graph of fig. 3, fall along the same curve, no matter which antagonist has been used, including atropine toward acetylcholine.

Correlation between R_{50} and k' . The equation of P obtained from equation I, defines a family of curves that are presented in fig. 2, where increasing values for the constant k' have been used in the calculation of each curve. It is easy to note that the R_{50} (intersection of each curve with the 50 per cent ordinate) is inversely proportional to the values of constant k' . This of course could be predicted from equation II, which can be deduced from equation I by making $P_0 = 0$ and $P = 50$, when $t = R_{50}$.

A strong indication of the validity of the method developed above has been shown through a statistical treatment of 93 pairs of values for R_{50} (found) and

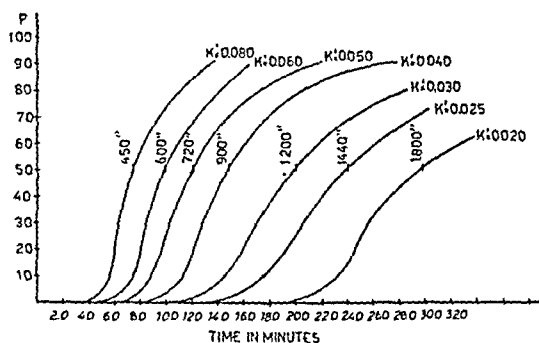


FIG. 2. THEORETICAL CURVES OBTAINED BY CHANGING IN EQUATION I THE VALUES FOR k' (INDICATED AT THE TOP OF EACH CURVE)

The values for R_{50} are indicated in seconds as the intersection of each curve with the 50 per cent ordinate.

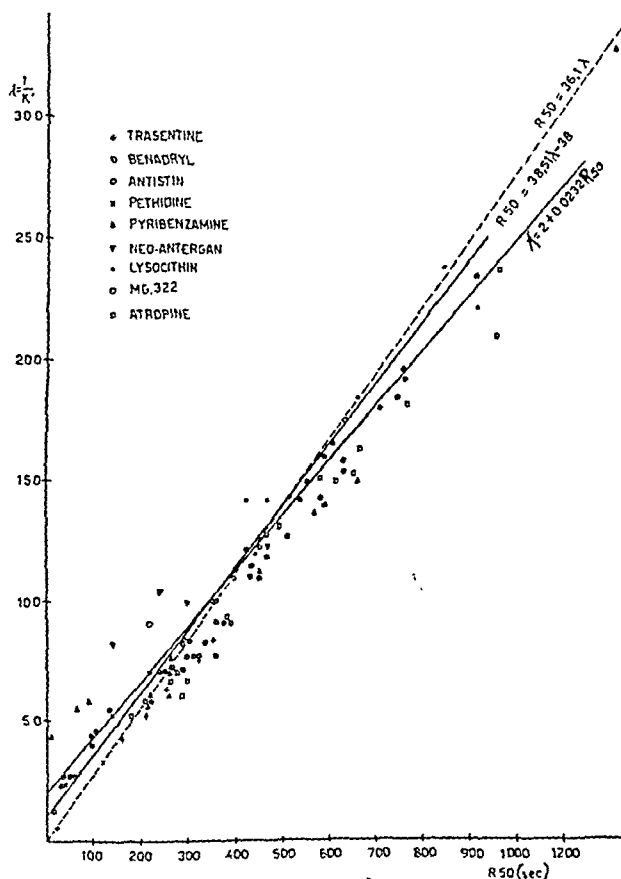


FIG. 3. CORRELATION BETWEEN λ ($= 1/k'$) AND R_{50} (FOUND) FOR 93 PAIRS OF VALUES FOR DIFFERENT ANTIHISTAMINICS AND DIFFERENT DOSES

The points for atropine are indicated in the plot but were not used for the computation of the regression coefficients indicated in the two equations that form the scatter diagram (full lines). The dotted line is the theoretical curve derived from equation II.

k' obtained for different inhibitors under different experimental conditions. Fig. 3 shows the scatter diagram, the theoretical curve (from equation II) and the experimental points obtained under quite different conditions. For simplicity, instead of constant k' as variable, we have taken its reciprocal ($\lambda = 1/k'$). The values of R_{50} (found) are indicated in seconds, in the abscissae. The points for atropine-acetylcholine are indicated in the plot but were not used for the calculation of the regression coefficients. The coefficient of correlation for k' and R_{50} (found) obtained with different doses of atropine is calculated in table 4.

The high correlation between k' and R_{50} (found), as indicated in fig. 3 and table 4, constitutes strong evidence that the principle underlying the method is sound since the values for both constants have been obtained by entirely independent

TABLE 4

Correlation between R_{50} (found) and k' for 93 pairs of values for different antihistaminics and different doses and 15 pairs for different doses of atropine

Antihistaminics:

$$\lambda = 1/k' \quad n = 93$$

$$\bar{x} = 10.5 \text{ (mean of } \lambda \text{)}$$

$$\bar{y} = 367'' \text{ (mean of } R_{50} \text{)}$$

$$S(xy) = 115753.0$$

$$S(x^2) = 3003.98$$

$$S(y^2) = 4,461,163.0$$

$$\text{Standard deviation of regression: } \pm 79''$$

$$\text{Coefficients of regression} \begin{cases} S(xy)/S(x^2) = 38.5 \\ S(xy)/S(y^2) = 0.023 \end{cases}$$

$$\text{Coefficient of correlation:}$$

$$r = S(xy)/\sqrt{S(x^2) \cdot S(y^2)} = 0.94$$

Atropine X acetylcholine:

$$n = 15$$

$$\bar{x} = 12.3 \text{ (means of } \lambda \text{)}$$

$$\bar{y} = 504'' \text{ (mean of } R_{50} \text{)}$$

$$S(x^2) = 512.85$$

$$S(y^2) = 991,401.0$$

$$S(xy) = 21,713.8$$

$$\text{Standard deviation of regression} = \pm 74''$$

$$\text{Coefficients of regression} \begin{cases} S(xy)/S(x^2) = 42.5 \\ S(xy)/S(y^2) = 0.022 \end{cases}$$

$$\text{Correlation coefficient:}$$

$$r = S(xy)/\sqrt{S(x^2) \cdot S(y^2)} = 0.97$$

methods. In fact, R_{50} (found) is read directly from the percentage-recovery plots, while k' has been defined as an average of all values calculated over the percentage responses data, being therefore indicative of the validity of equation I.

Effect of ions upon recovery. The method described has been highly useful in studying the effect of certain ions upon the phenomenon of recovery, as will be described shortly in another paper (3). The cations K^+ and Mg^{++} have been by far the most interesting, producing opposite effects, the former accelerating recovery and the latter slowing down the process of recovery. Although only assayed in very small concentrations, the cation Sr^{++} had a definite accelerating effect. The accelerating effect of Ca^{++} and Li^+ were less constant, depending upon circumstances that have not been mastered in our experiments. In several cases, calcium had a slight but definite slowing down effect while lithium had no effect at all. As shown in table 5, the effect produced by lithium was more marked when 5 microgm. of Benadryl were used as antagonist. When 1 microgm.

TABLE 5
Effects of ions upon recovery from Benadryl and atropine

NO. OF THE EXPERIMENT AND EFFECT	BENADRYL DOSE	CONC. OF IONS IN TYRODE SOL.	k'	"TESTS OF SIGNIFICANCE"*
	<i>microgm.</i>			
I Acceleration slowing down	5.0 (2 det.)	Standard	0.073	Between high K^+ and standard: $t = 5.9$ $P < 0.01$ H.S.
	5.0	$2 \times K^+$	0.147	
	5.0	$3 \times K^+$	0.120	Between $4 \times Mg^{++}$ and standard: $t = 2.3$ $P < 0.05$ S.
	5.0	$5 \times K^+$	0.180	
	5.0	$4 \times Mg^{++}$	0.055	
II Acceleration	1.0 (4 det.)	Standard	0.12	Between high Ca^{++} and stand.: $t = 1.9$ $P = 0.05$ S.
	1.0 (2 det.)	$4 \times Ca^{++}$	0.16	
III Acceleration	1.0 (3 det.)	Standard	0.11	Between high Ca^{++} and stand.: $t = 3.9$ $P < 0.01$ H.S.
	1.0 (2 det.)	$2 \times Ca^{++}$	0.13	
	1.0	$4 \times Ca^{++}$	0.22	
IV Acceleration	2.0 (3 det.)	Standard	0.11	Between high Li^+ and standard: $t = 1.0$ $P = 0.3$ N.S.
	2.0 (2 det.)	$1 \times Li^+$	0.10	
	2.0	$2 \times Li^+$	0.11	
V Acceleration	5.0 (2 det.)	Standard	0.08	Between high Li^+ and standard: $t = 7.9$ $P < 0.01$ H.S.
	5.0	$2 \times Li^+$	0.12	
VI Acceleration	1.0	Standard	0.11	Between ATP and standard: $t = 1.6$ $P < 0.2$ N.S.
	1.0	ATP ⁻	0.17	
VII Acceleration slowing down	10.0	Standard	0.063	Between Sr^{++} and standard: $t = 4.0$ $P < 0.01$ H.S. Between high Mg^{++} and stand.: $t = 2.7$ $P < 0.02$ S.
	10.0	$+0.0075 M Sr^{++}$	0.10	
	10.0	$3 \times Mg^{++}$	0.041	
VIII Acceleration	ATROPINE DOSE			
	<i>microgm.</i>			
	2.0	Standard	0.074	Between high K^+ and standard: $t = 6.3$ $P < 0.01$ H.S.
	2.0	$2 \times K^+$	0.210	
IX Slowing down	5.0	Standard	0.077	Between high Mg^{++} and stand.: $t = 2.7$ $P < 0.02$ S.
	5.0	$2 \times Mg^{++}$	0.055	

* Although there is no definite proof that variations of k' follow a normal distribution, application of the conventional methods for evaluating significance was tentatively done. For the calculation of the total variance, the sum of squares were pooled according to Snedecor (4). When the number of data available (n_1 and n_2) were very different and the variances also very different, the approximate method proposed by Cochran and Cox (see Snedecor, 4) was applied. The quantity t , as usual, was calculated by dividing the difference of the two means of k' by the square root of twice the variance of the means. It is to be insisted upon that the use of statistical methods to deal with data derived from a mathematical equation constituted only a first attempt to get more information from the data presented in the table. In that sense, information presented in column 5 will be no less conventional than that afforded by calculation of the SE for k' .

of Benadryl was used, acceleration produced by adding Li^+ to the Tyrode solution was probably non-significant. The anion ATP^- produced a slight acceleration of recovery.

As regards the constants described, an increase in potassium concentration produced a considerable increase in the value for the constant k' and consequently a decrease in the value for the constant R_{50} , with no alteration of the mathematical relationship existing between them (equation II). The point corresponding to the pair of values for both constants will run upward along the hyperbole representative of that equation. The opposite effect was observed when the concentration of magnesium was increased up to three to five times of that of the standard Tyrode solution. Table 5 shows typical experiments performed with Benadryl and atropine, showing the effects of different ions upon the values of k' .

The accelerating effect of potassium ions was very impressive. When their concentration was dropped to one-half of the standard, the muscle would even lose its capacity of recovery after inhibition by antihistaminics. The same inability to recover was also observed when the concentration of the calcium ions was dropped to one-half of the standard. If, however, the perfusing bath was switched to a richer potassium solution, recovery started very shortly and proceeded in an accelerating rate and in a few minutes the muscle would attain full sensitivity. That this full sensitivity was solidly acquired could be shown by changing again the bath to a one-half concentration of K^+ or Ca^{++} where the muscle continued to respond to the active drug as it did before, until after a new addition of the antagonist the sensitivity dropped to a very low level. A decrease in concentration of Mg^{++} to a half of the standard did not alter significantly the rate of recovery.

DISCUSSION. The process of recovery of the smooth muscle afforded a convenient method for testing drug antagonism. For each dose of the antagonist added to the perfusing bath one has a set of data to calculate the constant k' that is connected with the time for 50 per cent recovery through a simple mathematical equation. In the current methods for assaying antagonist potency only one datum is available for each addition of the antagonist dose. When R_{50} exceeds 600 seconds, at least seven figures are available for each dose of antagonist. With the current methods of assay, obtaining so many data would imply repetition of the assay with a considerable delay due to muscle recovery.

The theoretical implications of the method have been discussed in another paper (1). The quantitative analysis presented in this report led to the conclusion that inhibition by synthetic antihistaminics and by atropine toward acetylcholine is likely to depend upon the same intimate mechanism. Zadina (5) arrived at a similar conclusion, by using different methods of assay. Since inhibition by antihistaminics and atropine depends upon the same quantitative process, as shown by the calculation of constants k' and R_{50} , we are entitled to speak of identity and not merely of analogy. Moreover, cations as K^+ and Mg^{++} appeared to affect the course of recovery from inhibition by antihistaminics and atropine in a strikingly similar way. Another consequence of the analysis presented in this and the previ-

ous report is that recovery follows an auto-catalytic course. This conclusion was based on the fact that the values for constant k of a monomolecular reaction steadily increase as recovery proceeds. To calculate the constant k we have assumed that the percentage of blocked receptors ($100 - P_0$) at time 0, would measure the "concentration" (a) of a hypothetical complex formed between receptors and antagonist. Experimental evidence, however, indicated definitely that such a complex is not formed, and the process of recovery appeared to depend exclusively upon the intimate potentialities of the muscle itself. This unexpected conclusion opened up the theoretical problem of finding a new basis for the equations employed. Since recovery proceeds until the 100 per cent level is attained it seems logical to assume that $100 - P_0$ indicates some surface or spatial configuration that has been damaged or disturbed by the antagonist, the magni-

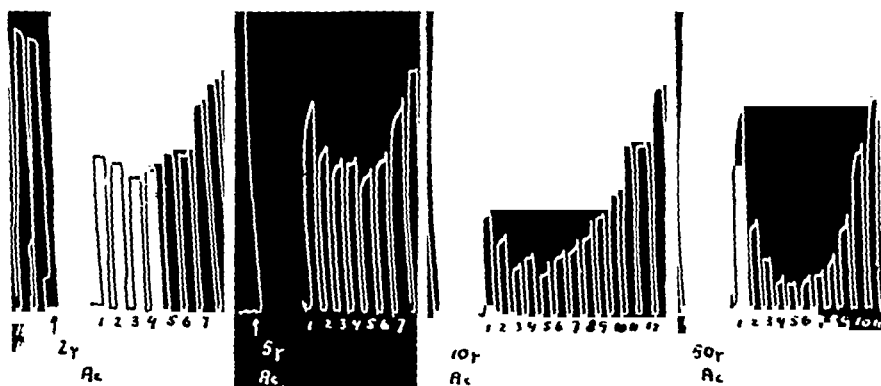


FIG. 4. REFRACTORINESS PRODUCED BY HIGH DOSES OF ACETYLCHOLINE

At the arrows, increasing doses (from 2 to 50 microgm.) of acetylcholine were applied for exactly one minute. In each case a maximal contraction followed (indicated by the open tracing). The muscle was assayed every 1.5 minute, with 0.15 cc. of histamine solution (1:2 million).

tude of that quantity denoting a sort of stress or pull conditioning velocity of recovery. The alternative explanation would be that recovery depends upon the auto-catalytic regeneration of an enzymatic system that has been damaged by the antagonist applied.

Another aspect of the problem should be discussed here. Refractoriness after high doses of spasmogenic agents such as histamine, acetylcholine and pilocarpine has been known for a long time (6). In 1946, Cantoni and Eastman (7) described the effect of ions of potassium upon refractoriness developed after the action of high concentrations of these agents. They interpreted their findings by assuming that refractoriness is the consequence of the exhaustion of a metabolite used up during the maximal contraction produced by the spasmogenic drug. We have verified that the curves of recovery after the interaction of high doses of histamine and acetylcholine are essentially the same as those that are typical for recovery after antihistaminics and atropine, with the difference that the process presents

two distinct phases. Immediately after the action of the high doses of the spasmogenic drug, the responses progressively decrease attaining a minimum; after that minimum is attained, the process of recovery begins and from there on, it follows the same law as for antihistaminics and atropine. The effect, however, depends strongly upon the actual concentration of the spasmogenic agent added. Although 1 or 2 microgm. of acetylcholine, for instance (fig. 4), are able to produce a maximal tetanic response, the refractoriness that follows that dose is rather slight in comparison with that produced by 10 or 50 microgm. of acetylcholine, that produce a similar tetanic response for exactly the same interval of time. The idea of an exhaustion of an energy yielding metabolite would not explain this quantitative difference. Moreover, by the action of antihistaminics and atropine, there occurs no response, notwithstanding that, refractoriness appears to depend upon an analogous mechanism. Since the effects of certain ions (potassium and magnesium) are similar in both processes, it might appear probable that refractoriness due to antihistaminics, atropine and also to high concentrations of histamine and acetylcholine would depend upon the same kind of disturbance of the ionic arrangement at the surface of the myosin molecule. Considerable work has been done on the effect of cations upon the physiological properties of myosin and interesting suggestions are contained in the monograph by Szent-Györgyi (8) on auto-catalytic disturbances of the ionic layers adsorbed upon the myosin molecule. A better understanding of the mechanism of recovery studied in this and in previous papers (1, 3) will probably depend upon progress in the study of ionic interaction during muscular contraction.

SUMMARY

Recovery of smooth muscle (guinea pig gut) after inhibition produced by antihistaminics and antispasmodics was used as an assaying method for quantitative evaluation of antagonism potency, using histamine as spasmogenic drug and Benadryl as a running control (potency = 1). The constant k' and the index R_{50} (time in seconds for a 50 per cent recovery) were employed as numerical data to evaluate potency of the antagonists employed (Benadryl, Neo-antergan, Pyribenzamine, Antistin, Mg.322, Trasentine and Pethidine).

Recovery from inhibition produced by atropine toward acetylcholine was found to follow the same numerical laws as recovery from inhibition toward histamine, by the above mentioned inhibitors.

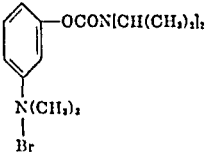
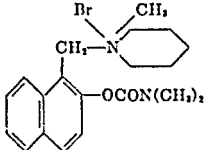
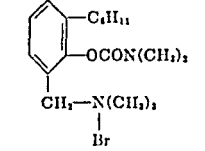
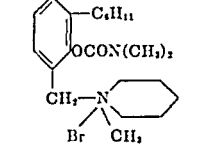
Correlation between the two quantities k' and R_{50} (found) as obtained by entirely independent procedures, was found to be highly significant ($r = 0.94$ for $n = 93$).

Using the method of recovery, the effect of increasing the concentration of cations (calcium, potassium, magnesium, lithium and strontium) in the standard Tyrode solution was studied. The cations K^+ and Mg^{++} displayed strikingly antagonistic effects, the former accelerating the process of recovery and the latter slowing it down.

REFERENCES

1. ROCHA E SILVA, M., AND BERALDO, W. T.: *THIS JOURNAL*, 93: 457, 1948.
2. CAVALLINI, G.: *Il Farmaco*, 2: 89, 1947.
3. ROCHA E SILVA, M., AND BERALDO, W. T.: *Science*, 108: 162, 1948.
4. SNEDECOR, G. W.: *Statistical Methods*. The Collegiate Press, Inc., Ames, Iowa, 1946.
5. ZADINA, R.: *Proc. XVIIth Internat. Cong. Physiol.*, Oxford, 169, 1947.
6. CLARK, A. J.: In *Heffter's Handbuch exper. Pharmakol. Ergaenz.*, 4: J. Springer, Berlin, 1937.
7. CANTONI, G. L., AND EASTMAN, G.: *THIS JOURNAL*, 87: 392, 1946.
8. SZENT-GYÖRGYI, A.: *Chemistry of muscular contraction*. Acad. Press, New York, 1947.

TABLE 1—Continued

Nu 1331		Di-isopropylcarbamate of 3-hydroxyphenyl - trimethyl - ammonium bromide
Nu 1581		Dimethylcarbamate of (2-hydroxy-1-naphthyl) methyl methylpiperidinium bromide
Nu 906		Dimethylcarbamate of (2-hydroxy-3-cyclohexylbenzyl) trimethylammonium bromide
Nu 911		Dimethylcarbamate of (2-hydroxy-3-cyclohexylbenzyl) methyl methylpiperidinium bromide

Nu 906 and Nu 911 when 1 per cent solutions were used. It seemed that head-drop was about to occur, but the characteristic state was not established. On the assumption that excretion or inactivation was occurring at a rate comparable to that of injection, the concentration was increased to 5 per cent. Head-drop then was produced which was of short duration and was uncomplicated by muscarinic side effects or fasciculation. In this series of compounds, the dose necessary to produce paralysis increased as the muscarinic action decreased.

The substances are listed in table 2 B in decreasing order of their capacity to antagonize curare. When prior intravenous injections of the neostigmine-like agents into rabbits were followed by d-tubocurarine assays, a definite capacity to increase the dose of d-tubocurarine required to produce head-drop could be demonstrated for all compounds except Nu 906 and Nu 911 (table 2). Only in the instance of neostigmine was it possible to find a dose which increased the curare head-drop dose to more than 200 per cent of the control value. This suggests

that there may be a quantitative limit to the antagonism of curare, determined by the number of available molecules of cholinesterase or of acetylcholine present in the region of the neuromuscular units affected under the conditions of the assay.

An attempt was made, for the purposes of comparison, to find a dose of each of the neostigmine-like compounds which would increase the curare assay titre to approximately 175 per cent of control. For nearly every drug having both actions, this dose was about one-fourth that necessary for the production of head-drop by the compound administered alone.

TABLE 2
Curare and anti-curare action of carbamate compounds

	NU COMPOUND									
	Neostigmine	1526	683	1317	658	1560	1331	1584	906	911
<i>A. Curare action—rabbits head-drop</i>										
Dose mgm./kgm.....	0.122	0.14	0.23	1.3	2.7	5.5	10.5	33.5	20.80	11.67
<i>B. Anti-curare action—antagonism of d-tubocurarine</i>										
DOSE OF COMPD.	PER CENT OF CONTROL HEAD-DROP DOSE D-TUBOCURARINE/NUMBER OF ANIMALS TESTED									
0.025	184/10	144/4	115/5							
0.05	256/10	193/11	140/9							
0.075	263/9									
0.1			167/10			109/2	95/2		100/2	
0.2			199/10			131/9	108/2		103/3	
0.25				184/6	135/8					
0.5				181/6	152/8	150/10	106/2	103/2	104/2	
0.75					179/9					
1.0						183/12	123/4	126/8	102/2	105/2
2.0							165/15	153/10	102/2	100/4
5.0						160/8		176/10		100/2
5.8									82/2	

It seemed desirable to establish the synergistic curarizing action of neostigmine, the strongest anti-curare drug, with the compounds of the series having the purest curare-like action, Nu 911 and Nu 906. Rabbits given prior intravenous injections of neostigmine (0.0125 mgm./kgm., 2 rabbits; 0.05 mgm./kgm., 5 rabbits; and 0.075 mgm./kgm., 2 rabbits) were titrated to head-drop with Nu 911. The head-drop dose of this curare-like neostigmine congener was lowered by the prior injection of neostigmine to 53 per cent, 50 per cent, and 37 per cent, respectively, of the control value. The animals showed no signs of muscarinic action, had moderate respiratory embarrassment and recovery from head-drop within 40 to 90 seconds. Head-drop could again be produced if additional doses of Nu 911 were given. The same relationship to neostigmine was established in preliminary experiments with Nu 906.

DISCUSSION. We have described a series of carbonyl compounds which range from congeners having strong anti-curare activity to compounds which have curare-like action only. It is of significance that if the members of this series be arranged in order of their decreasing anti-curare potency as determined in rabbits, this order very closely coincides with the order of their cholinergic action.

The synergism in curare-like activity between Nu 911 and neostigmine is reminiscent of the results obtained when similar doses of neostigmine were given prior to assays of quinine ethochloride as a curarizing agent (2). With quinine ethochloride, smaller doses of neostigmine were antagonistic to and the larger doses summated with its paralytic action. The synergism between neostigmine and Nu 911 and Nu 906 raises the possibility that the curare-like action of neostigmine here is exaggerated by the presence of molecules of a compound of similar structure. The two actions, the anti-curare and the curare-like, may be due to different chemical properties. In abolishing the anti-curare action in this series, the curare action was correspondingly weakened, but, nevertheless, persisted in demonstrable amount. Even though relatively large quantities of Nu 906 and Nu 911 were necessary to produce curare-like effects, it is possible that compounds of similar structure might be more potent paralyzants and yet be as free of objectionable cholinergic side actions.

CONCLUSIONS

A series of neostigmine congeners has been studied by means of the rabbit head-drop assay for curare and has been demonstrated to have both curare and anti-curare action.

Two compounds, Nu 911 and Nu 906, were shown to be purely curare-like. This curare-like action differs from the true curare action in that it is summated with, not antagonized by, neostigmine.

REFERENCES

1. LEHMANN, G.: Jubilee Volume Dedicated to Emil Borell (Hoffman-La Roche, Inc.), Frederick Reinhardt, Ltd. Co., Basle, p. 314.
2. CHASE, H. F., SCHMIDT, J. L., AND BHATTACHARYA, B. K.: *THIS JOURNAL*, 91: 236, 1947.

THE COMPARATIVE MUSCLE-PARALYZING ACTIVITY OF SOME SUBSTITUTED GLYCEROL ETHERS¹

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While engaged in a toxicity study concerning the potential industrial hazards of substituted glycerol ethers, we observed (1) that paralysis could be produced in mice, rats, and rabbits by the oral administration of several beta and gamma derivatives of alpha glycerol ethers. This was contrary to the findings of Bradley and Berger (2) who stated that the grouping $\text{—O—CH}_2\text{—CROH—CR}_2\text{—OH}$ appeared to be necessary for the production of paralysis. Recently, Berger (3), reporting on a more extensive series of glycerol ether derivatives, re-emphasized the point that substitution in the beta and/or gamma hydroxyl group decreased or destroyed the paralyzing activity of the alpha glycerol ethers. Since a large series of alpha and alpha, gamma substituted ethers and esters were available to us, we undertook the evaluation of the relative paralyzing activity of these compounds in mice and compared them with Myanesin, the most successful compound of Berger's series.

Berger has based his comparison of the relative effectiveness of glycerol ethers solely on their ability to produce paralysis in small quantities. However, it seemed to us that other properties were also important in agents which might be used to produce muscular relaxation in man. We, therefore, included a determination of the duration of paralysis and of the solubility characteristics in the comparison of the members of our series.

I. PARALYSIS IN MICE PRODUCED BY SUBSTITUTED GLYCEROL ETHERS

METHOD. Paralyzing² and lethal doses were determined by intraperitoneal injection of a solution or suspension of each compound in mice. The intraperitoneal route was chosen in preference to other routes, since results obtained by this method were more constant. Male, white, inbred Swiss mice, five to six weeks old, weighing 20 ± 2 gm. were used exclusively. Animals were denied food ten to fourteen hours prior to injection. Each compound was prepared immediately before use in 5, 10 and 20 per cent concentrations, w/v or v/v, depending on its physical state. When solubility permitted, 0.9 per cent NaCl solution was the solvent; otherwise, a 6.0 per cent gum acacia homogenate was prepared. Injected mice were tested for loss of the righting reflex at one-minute intervals. More frequent testing was found to stimulate the mice and delay the onset of paralysis. In those cases in which paralysis occurred it commenced within eight minutes of injection. Paralyzed animals were observed until recovery or death resulted.

The number of paralyzed and dead animals, the time for loss and recovery of the righting reflex, and the time of death were recorded. Using the probit method of Miller and Tainter

¹ Aided by grants from the Research Board of the University of California Medical School and the Lilly Research Laboratories, Indianapolis 6, Indiana.

² Paralysis was said to have occurred when a mouse placed on its back failed to right itself for at least 60 seconds.

(4), the LD_{50} and PD_{50} were estimated and the activity ratio, (LD_{50}/PD_{50}), and the standard error calculated.

RESULTS. The pharmacologic effects obtained were qualitatively similar for all classes of compounds. The outstanding sign was a flaccid paralysis, which was entirely reversible at small doses. There was no excitatory phase preceding paralysis, and the onset was usually rapid. Weakness of the hind extremities and impaired locomotion usually occurred about 30 seconds prior to loss of the righting reflex. Respirations were depressed and, with higher doses, became diaphragmatic in nature. When death occurred, it appeared to be due to respiratory paralysis. These observations are similar to those made by Berger and Bradley (5) and others (6).

The muscle-paralyzing activity levels of 43 members of eight different classes of substituted glycerols was determined. These classes included the following: alpha ethers; alpha, gamma diethers; alpha, beta, gamma triethers; gamma esters of alpha ethers; beta, gamma esters of alpha ethers; alpha, gamma esters of beta ethers; beta esters of alpha, gamma diethers; and alpha glycidyl ethers.

Quantitatively, the compounds were found to differ in (a) the time of onset of paralysis, (b) the duration of paralysis, (c) the quantity necessary to produce paralysis, (d) the ratio between the lethal and paralyzing doses. These differences are shown in table 1 for certain representative compounds.

The following compounds were found unsatisfactory, since they possessed one or more of the following undesirable characteristics: (1) they were incapable of producing paralysis in doses of less than 0.66 gm./kgm., (2) the LD_{50}/PP_{50} was less than 2.0, (3) they were completely water-insoluble. Compounds which fell into this category grouped according to chemical class were: (1) alpha glycerol ethers: methyl; ethyl; isopropyl; secondary butyl; tertiary butyl; amyl; 3,5,5 tri-methyl cyclohexyl; methyl amyl carbonyl. (2) alpha gamma diethers: methyl, methyl; isopropyl, isopropyl; isoamyl, isoamyl; diethyl carbonyl, diethyl carbonyl; methyl isobutyl carbonyl, methyl isobutyl carbonyl; methyl, phenyl. (3) alpha beta gamma triethers: methyl, methyl, methyl; methyl isobutyl carbonyl, beta hydroxy ethyl, methyl isobutyl carbonyl. (4) beta, gamma diesters of alpha ethers: secondary butyl, acetate, acetate; cyclohexyl, acetate, acetate; tri-methyl cyclohexyl, acetate, acetate. (5) alpha gamma diether beta esters: methyl isobutyl carbonyl, acetate, methyl isobutyl carbonyl; phenyl, acetate, methyl. (6) alpha gamma diesters of beta ethers: acetate, isopropyl, acetate. (7) glycidyl ethers: isopropyl; phenyl; nonyl phenyl.

II. PHYSICAL PROPERTIES OF THE SUBSTITUTED GLYCEROL ETHERS. With the exception of the alpha ortho tolyl derivative, the alpha phenyl glycerol ether, and the beta sodium succinate salt of alpha ethyl gamma phenyl glycerol ether, all of the compounds studied were liquids. The majority of the compounds possessed but slight water solubility, and consequently had to be administered as suspensions in gum acacia. The partition coefficients of the compounds which showed satisfactory activity ratios were determined in an effort to obtain addi-

* Adapted from the range finding toxicity determination of Deichmann and Mergard (7).

CLASS OF COMPOUND		PD ₅₀ IN GR. ± SE	LD ₅₀ IN GR. ± SE	PD ₅₀ IN MM	LD ₅₀ IN MM	RATIO LD ₅₀ PD ₅₀	TIME FOR ONSET OF PARALYSIS IN MINUTES ± SD		MEAN DURATION OF PARALYSIS IN MINUTES ± SD	
							PD ₁₀	LD ₁₀	PD ₅₀	LD ₅₀
Alpha gly- cerol ethers	n-Butyl	0.51 ± .04	1.30 ± .03	3.4	8.8	2.6	1.2 ± 0.0	1.0 ± 0.0	3.8 ± 1.6	43.0 ± 14.4
	Cyclohexyl	0.49 ± .02	1.14 ± .05	2.8	6.6	2.3	2.6 ± 0.8	1.0 ± 0.5	4.0 ± 1.2	37.3 ± 20.0
	Phenyl	0.42 ± .04	1.28 ± .03	2.5	7.6	3.1	2.7 ± 0.8	1.3 ± 0.3	7.3 ± 3.7	132.0 ± 32.2
	"Myanesis" (o-tolyl)	0.20 ± .01	0.60 ± .02	1.1	3.3	3.0	2.4 ± 0.6	0.8 ± 0.2	4.5 ± 2.9	89.0 ± 25.0
Alpha gamma di-glycerol ethers	Ethyl phenyl	0.22 ± .01	0.59 ± .04	1.1	3.0	2.7	1.5 ± 0.0	1.0 ± 0.0	1.7 ± 0.9	60.0 ± 11.0
	Isopropyl phenyl	0.20 ± .04	0.53 ± .03	1.0	2.5	2.6	2.5 ± 1.0	1.0 ± 0.0	2.5 ± 1.0	19.8 ± 5.0
	n-Butyl phenyl	0.28 ± .01	0.86 ± .02	1.2	3.8	3.1	3.9 ± 1.2	2.2 ± 0.9	12.5 ± 6.2	49.4 ± 17.0
	Isopropyl tolyl	0.21 ± .03	0.59 ± .01	0.9	2.7	2.9	4.0 ± 0.1	2.1 ± 0.5	7.0 ± 1.2	91.3 ± 36.0
	Ethyl isopropyl	1.07 ± .09	3.18 ± .11	6.6	19.6	3.0	2.4 ± 1.4	1.1 ± 0.1	14.0 ± 1.0	240.0 ± 86.0
	Di-n-butyl	0.35 ± .03	1.01 ± .01	1.7	5.0	2.9	3.8 ± 1.4	3.1 ± 2.3	7.1 ± 1.8	46.0 ± 9.3
Esters of glycerol ethers	Phenyl mono acetate	0.33 ± .01	1.36 ± .00	1.6	6.5	4.2	2.5 ± 0.7	1.0 ± 0.0	2.4 ± 1.3	119.0 ± 48.0
	Phenyl diacetate	0.64 ± .03	1.85 ± .06	2.5	7.4	2.9	6.0 ± 0.9	2.2 ± 0.4	23.9 ± 15.0	246.0 ± 48.0
	Tolyl diacetate	0.56 ± .02	1.44 ± .01	2.1	5.4	2.6	6.0 ± 1.7	3.7 ± 0.1	28.0 ± 15.3	95.0 ± 26.0
	Di-n-butyl mono ace- tate	0.69 ± .00	1.93 ± .00	2.8	7.8	2.8	5.4 ± 2.4	3.0 ± 1.3	20.4 ± 10.2	122.0 ± 21.0
	Phenyl ethyl Na suc- cinate	0.54 ± .02	1.38 ± .08	1.6	4.1	2.6	6.5 ± 2.3	2.9 ± 0.5	15.4 ± 3.3	117.0 ± 52.0

† These compounds were supplied by the Organic Synthesis Department, Shell Development Laboratories, Emeryville, California.

tional physical data which might help to explain differences in pharmacologic activity.

METHOD. Solutions of the glycerol derivatives were made up in either distilled water or USP cottonseed oil, depending upon the relative solubility of the compounds. To this solution in water or oil, oil or water was added, the mixture was shaken, and it was placed in a water bath at 25° C. until the phases had separated. The refractive index of the oil or

TABLE 2
*Summary of important physical data for certain glycerol ethers**

SUBSTITUTED GLYCEROL ETHER	MOLECULAR WEIGHT	SPECIFIC GRAVITY*	APPROXIMATE WATER SOLUBILITY†	K = $\frac{\text{oil}}{\text{water}}$
			<i>per cent</i>	
Alpha n-butyl.....	148	1.0007	100.0	0.041
Alpha phenyl.....	168	1.148‡	10.0	0.17
Alpha cyclohexyl.....	174	1.0680	1.0	0.10
Alpha o-tolyl (Myanesin)....	182	1.1400‡	1.0	1.50
Alpha ethyl, gamma iso-propyl.....	162	0.9291	100.0	2.85
Alpha ethyl, gamma phenyl...	196	1.0730	0.1	13.7
Alpha isopropyl, gamma phenyl.....	210	1.0137	<0.1	45.5
Alpha n-butyl, gamma phenyl.	224	1.0003	<0.1	35.7
Alpha isopropyl, gamma tolyl	223	1.0269	<0.1	294.0
Alpha ethyl, beta sodium succinate gamma phenyl ..	338	—	20.0	0.0032
Alpha phenyl, gamma acetate.	210	1.1633	0.1	16.4
Alpha phenyl, beta, gamma di-acetate.....	252	1.1501	<0.1	71.4
Alpha tolyl, beta, gamma di-acetate	266	1.1252	<0.1	154.0
Alpha, gamma dibutyl, beta acetate	246	0.9524	<0.1	333.0

* The authors acknowledge the help of the Physical Chemistry Department, Shell Development Laboratories, in obtaining these data.

* In vacuum at 20°/4°C. unless otherwise noted.

† Expressed to nearest unit of 10.

‡ In vacuum at 60°/4°C.

water phase was determined by means of an Abbé refractometer and the concentration of the ethers in the two phases was determined by reference to standard concentration curves.

In table 2 appears essential physical data concerning the more active derivatives.

DISCUSSION. Comparison of our findings with those of Berger concerning the relative potency of identical glycerol ethers may show discrepancies because of the difference in the route of administration employed. However, similar trends are detectable with both methods. For example, while we found the butyl and not the amyl derivative to be the most potent in the mono-substituted alpha

ethers, both investigations indicated that neither aliphatic ether was as potent as were the aryl substituted derivatives. Our differences in potency and activity ratio between the phenyl mono ether and the ortho methyl phenyl ether were not as great as reported by Berger. Also, in our series the mono cyclohexyl ether produced a satisfactory paralysis without convulsions. The greatest difference in our conclusions, however, was as to the grouping necessary for the production of satisfactory paralysis. Contrary to Berger's opinion that "substitution in the hydroxy groups also decreased or destroyed activity," we found that the substitution of an alkyl group in the gamma hydroxy position of several of the mono glycerol ethers increased the potency of the alpha ethers to the point where it coincided with that of Myanesin. Esterification of the free hydroxy groups had a varying effect upon potency. In some instances it was increased, while in others it was decreased. The outstanding effect of esterification was the increase in the duration of paralyzing activity. Duration of activity has not been used as a criterion of comparison by Berger in evaluating the potential usefulness of his series of substituted ethers.

We believe that duration of action is of considerable importance in choosing agents for further testing of effectiveness in the production of muscular paralysis. We have arbitrarily chosen the PD_{50} and LD_{50} doses as convenient points for measuring the duration of action. Any other dose level might serve as well. The determination of paralyzing doses, lethal doses, their ratios, and the duration of paralysis by this mouse technic furnishes an objective method of comparing two or more compounds. However, these data must be interpreted with caution, since the picture of "paralysis" in the mouse is not well delineated. With certain of the compounds we have tested, it is our impression that there was a considerable degree of sensory depression accompanying the muscular relaxation. This was especially noticeable with the esters at the higher dose levels.

The solubility characteristics of the ethers should be considered in making the selection of agents for further evaluation. This is especially important if the compound is to be administered orally. Myanesin, for example, because of its poor water solubility (one per cent), does not possess as high an activity ratio following oral administration to mice and rats as it possesses following parenteral administration. The distribution coefficient between oil and water, $K\left(\frac{\text{oil}}{\text{water}}\right)$, is also a factor of importance when cellular penetration and duration of action are vital. Reference to table 1 and table 2 indicates that a majority of compounds which have a prolonged action also possess a high $K\left(\frac{\text{oil}}{\text{water}}\right)$ value.

The alpha ethyl gamma isopropyl derivative is unique in that it is the only water soluble compound in this series which possesses a higher K value than Myanesin. While the potency of the ethyl isopropyl derivative is considerably less than that of Myanesin, the activity ratios are comparable, and the longer duration of action obtained with the double ether suggests that it would be advisable to examine it more extensively.

While on theoretical grounds it would seem advantageous to explore the

lipoid solubility characteristics of the alpha gamma diethers and esters, many of these compounds are difficult to administer because of their extremely low water solubility. An attempt was made to solubilize one of these agents by preparing the double salt of an easily metabolized organic acid. The results obtained were partially successful, since the solubilized ether possessed double the duration of action but was only slightly less potent. It is interesting to note that while the latent period of the acid derivative was definitely longer, paralysis did occur in a reasonably short time, indicating a rapid metabolism of the solubilizing group. Further exploration along these lines is indicated.

SUMMARY

1. A series of substituted glycerol ethers and esters were examined for their muscle paralyzing activity.

2. Contrary to the conclusions of Berger, we have found considerable paralyzing activity to be present in alpha gamma substituted glycerol ethers and in the mono and diesters of alpha ethers.

3. A general correlation was shown to exist between lipoid solubility and duration of action.

4. We believe that physical properties and duration of action should be considered in addition to potency and activity ratios when evaluating new compounds of this class.

5. The alpha ethyl gamma isopropyl derivative, because of its water solubility and duration of action, warrants further pharmacologic study.

REFERENCES

1. HINE, C. H., AND CHRISTENSEN, H. E.: *Fed. Proc.*, 7: 254, 1948.
2. BRADLEY, W., AND BERGER, F. M.: *Nature*, 159: 813, 1947.
3. BERGER, F. M.: *THIS JOURNAL*, 93: 470, 1948.
4. MILLER, L. C., AND TAINTER, M. L.: *Proc. Soc. Exper. Biol. and Med.*, 57: 261, 1944.
5. BERGER, F. M., AND BRADLEY, W.: *Brit. J. Pharmacol.*, 1: 265, 1946.
6. EVERETT, G. M., AND RICHARDS, R. K.: Personal communication.
7. DEICHMANN, W. B., AND MERGARD, E. G.: *J. Ind. Hyg. and Toxicol.*, 30: 373, 1948.

THE REFLEX RESPIRATORY AND CIRCULATORY ACTIONS OF VERATRIDINE ON PULMONARY, CARDIAC AND CAROTID RECEPTORS^{1, 2}

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Although the circulatory effects of veratrum and its various alkaloidal active principles have been investigated quite extensively (3) the respiratory effects have never been studied systematically. This is not surprising since the most definitive studies of veratrum actions have been made with perfusion, heart-lung or open chest preparations. Yet the respiratory effects are remarkable enough to warrant further study. The general pattern—respiratory depression or apnea associated with bradycardia and vasodilatation, all abolished by vagotomy and therefore presumably reflex in origin—is almost unique among drugs and is sometimes, in honor of its discoverer (4), called the Bezold effect. This effect duplicates that of strong stimulation of the pressoreflex system of the carotid sinuses, but the influence of vagotomy proves that the actions of veratrum do not depend on carotid reflexes, which are characteristically intensified by vagotomy (5, 6). Veratrum apnea furthermore is exceptional among chemoreflexes, which otherwise are almost invariably stimulant to respiration. Finally according to existing information from experiments on cats (7) the receptors responsible for veratrum apnea are located in the distribution of the pulmonary artery while those from which the circulatory effects arise are supplied by the coronaries. Apart from the general interest of this distribution of the veratrum-sensitive nerve structures, this is the first hint of the presence of chemoreceptors in the distribution of the pulmonary artery, where their physiological significance, if any, would necessarily be entirely different from that of chemoreceptors supplied with arterialized blood.

The experiments now to be described were undertaken in hopes of securing more complete information about the respiratory effects of veratrum, in an animal (the dog) not previously studied and under conditions as close as possible to the normal.

METHODS. Mongrel dogs weighing from 9 to 19 kgm. were used. Anesthesia was produced by morphine (1 mgm. per kgm. subcutaneously) and chloralose (50–100 mgm. per kgm. intra-

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vénously). A tracheal cannula was used routinely. Blood pressure was recorded from a femoral artery by a mercury manometer. Respiratory movements were registered by a pneumograph and tambour. Injections were made (a) intravenously into an external jugular; (b) into the coronary arteries through a catheter introduced through one common carotid artery and manipulated so as to bring the injecting tip either proximal or distal to the origin of the coronaries; (c) into the pulmonary artery through a catheter inserted through an external jugular vein and manipulated under fluoroscopic control so as to bring its tip into the pulmonary artery at various levels; (d) into the carotid sinus (pressoreceptor) or carotid body (chemoreceptor) regions by means of injection needles inserted into

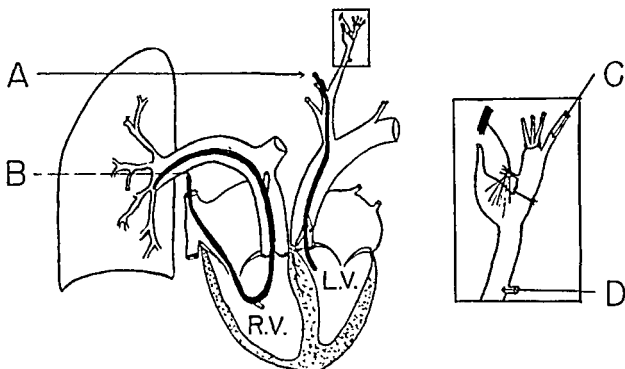


FIG. 1. ARRANGEMENT OF INJECTING DEVICES

A catheter introduced through one common carotid artery with injecting tip either proximal or distal to the origin of the coronaries; B. catheter introduced through an external jugular vein with its tip into the pulmonary artery at various levels; C. cannulated lingual artery for injection into the carotid body chemoreceptors; D. cannulated superior thyroid artery for injection into the carotid sinus pressoreceptors.

the superior thyroid and lingual arteries with the external carotid tied between the origins of the internal carotid and occipital arteries (8). The arrangement of the various injecting devices is shown in fig. 1. Further details of the means by which the site of injection was ascertained during the experiment will be given in conjunction with the results obtained. Veratridine was used to elicit the Bezold effect because Krayer⁶ and his associates (9, 10) have found it one of the most effective of the veratrum alkaloids in eliciting the full reflex.

RESULTS. I. An intravenous injection of a suitable dose of veratridine was followed in about six to eight seconds by the typical Bezold effect, viz., apnea, bradycardia and hypotension (fig. 2). The minimum effective dose was usually 1 microgm. per kgm., which produced an apnea lasting about fifteen seconds,

⁶ The dogs which were subjected to cardiac catheterization were given sodium heparin, 100 Toronto unit/kgm.

⁷ We wish to thank Dr. Otto Krayer, Harvard Medical School, for supplying us with the purified veratrum alkaloids.

heart rate changes ranging from slight slowing to complete asystole, and a fall in mean blood pressure to about 70 per cent of the pre-injection level. Smaller doses produced hypotension but there was little or no bradycardia; the degree of respiratory depression tended to parallel the bradycardia. Atropine selectively eliminated the bradycardia but the hypotension and respiratory depression remained. Bilateral vagotomy abolished all effects. Section of the carotid sinus and depressor nerves (the latter, following Koch (11) by cutting each superior laryngeal nerve close to its parent vagus so as to retain the pulmonary afferents) produced no notable change in the veratridine effect except that the hypotension lasted a longer time.

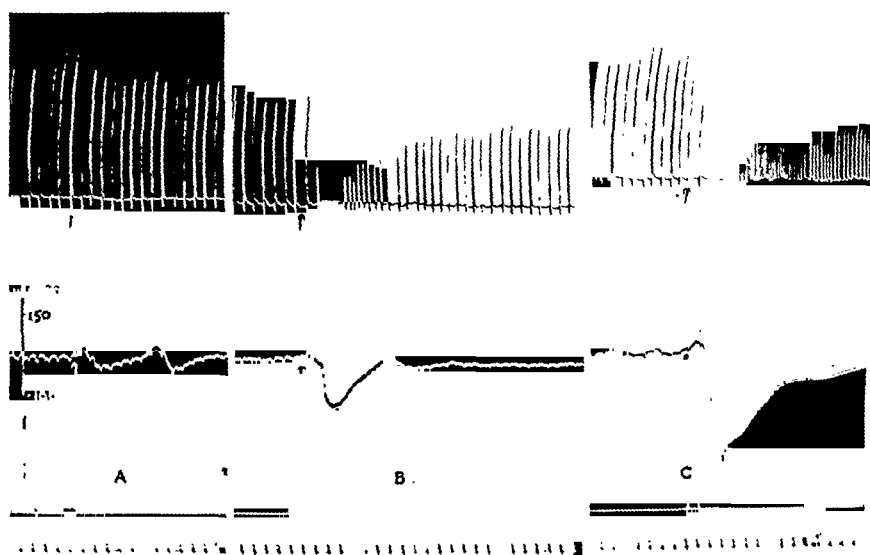


FIG. 2. INCREASING INTRAVENOUS DOSES OF VERATRIDINE

A. 0.5 microgm./kgm.; B. 1.0 microgm./kgm.; C. 1.5 microgm./kgm. Record of respiration, arterial blood pressure, signal, and time interval of 5 seconds. Dog, 16 kgm.

The phenomenon of tachyphylaxis, already well-known for veratrum alkaloids (12, 13) proved to be a major obstacle in these experiments. Drawing on previous experience with ephedrine (14), with which tachyphylaxis can be minimized by employing minimal dosages and allowing time to elapse between them, we used the minimum effective dose of veratridine so far as possible, and paused at least five minutes between injections. Even then we found that after three to ten such injections there was a progressive decrease and disappearance of effects in the following order: apnea, bradycardia and hypotension. Eventually even large doses became ineffective in changing the respiration and pulse although hypotension was still produced.

The intensity of the respiratory depression produced by veratridine can be judged from the fact that apnea was still elicited when the drug was injected during the hyperpnea produced by inhalation of 5 per cent CO_2 in oxygen or of

6 per cent oxygen in nitrogen, or by intravenous injection of 0.01 mgm. of methacholine per kgm. The state of the circulation also plays a role: in an animal in shock an intravenous injection of 3 mgm. of veratridine per kgm. produced only slight hypotension and no apnea, but after restoration of the circulation by fluid replacement the full response (including apnea) returned.

II. *Localization of the receptors.* A. *In the heart.* For these experiments injections were made through a catheter introduced through a common carotid (fig. 1-A). The position of the tip in relation to the origin of the coronary arteries was ascertained by injection of acetylcholine through the catheter. This produced immediate asystole if injected so as to enter the coronary stream, a more gradual hypotension without bradycardia if injected into the aorta beyond the coronaries (fig. 3). Veratridine under the former circumstances caused bradycardia and hypotension while under the latter it had no distinct effect. In seven of the twelve dogs thus studied respiration was not visibly changed, while in the remaining five there was a slight decrease in height of the pneumogram following injection of veratridine into the coronary stream. In no case was there any apnea although an intravenous injection producing equal or smaller circulatory responses regularly gave rise to definite apnea (fig. 4). Thus the apnea is seen not to be due to irradiation into the respiratory center of strong impulses intended primarily for the cardioinhibitory center, and a totally separate set of receptors is probable. These are not in the aortic or carotid bodies since injections of veratridine into the aortic arch distal to the origin of the coronaries were ineffective even when six times the minimum effective dose was used and immediate hyperpnea following injection of sodium cyanide indicated accessibility of this set of chemoreceptors to drugs injected through the catheter (fig. 3).

Are the receptors responsible for bradycardia and hypotension located on the endocardial surface? Evidence on this was sought by measuring the latent period⁷ between injection of veratridine and the onset of bradycardia when the injections were made (a) into the left auricle (through a tube previously inserted through the auricular appendage) and (b) into the left ventricle (by pushing the aortic catheter through the aortic valves). This period averaged 3.9 seconds following (a), 2.3 seconds following (b). The receptors therefore appear not to be on the endocardial surface, but farther in the course of the blood and thus in the distribution of the coronary arteries.

B. *Pulmonary receptors.* Injections into the right side of the heart or into the pulmonary artery, by means of a suitable catheter (fig. 1-B), elicited essentially the same responses as intravenous injections. The circulatory effects were almost equal to those of injections into the coronaries but here apnea also was seen (fig. 5). When the catheter tip was in one of the distal branches of the pulmonary artery veratridine produced an immediate apnea while the bradycardia came on about six seconds later. Blocking the ipsilateral vagus with cold or procaine abolished the apnea of such injections but the circulatory effects were unchanged or only slightly diminished (fig. 6). That this loss of the apneic response was

⁷ An electrocardiograph facilitated the measurement of the cardiac reflex latent period to the nearest 0.2 second.

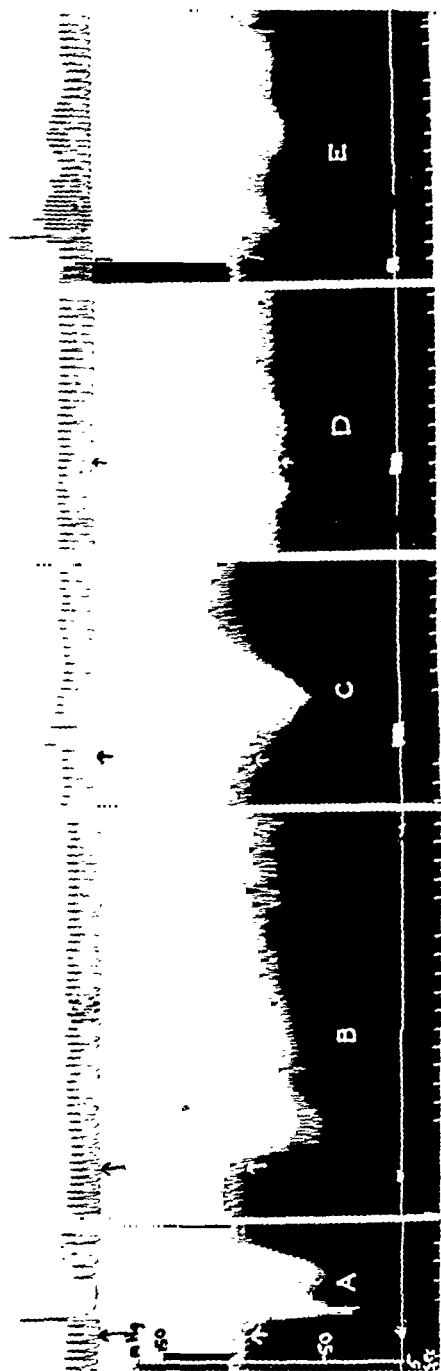


FIG. 3. INJECTIONS PROXIMAL AND DISTAL TO ORIGIN OF CORONARIES

A. acetylcholine 0.5 mgm./kgm. and B. veratridine 1 microgm./kgm. into left ventricle. Same doses of C. acetylcholine and D. veratridine and E. sodium cyanide 0.5 mgm./kgm. into aortic arch. Record of respiration, arterial blood pressure, signal, and time interval of 5 seconds. Dog, 17 kgm.

not simply due to tachyphylaxis was shown by its return on warming the cold-blocked vagus and its retention when the contralateral vagus was blocked. Thus receptors in the peripheral parts of the lungs are seen to be mainly respon-

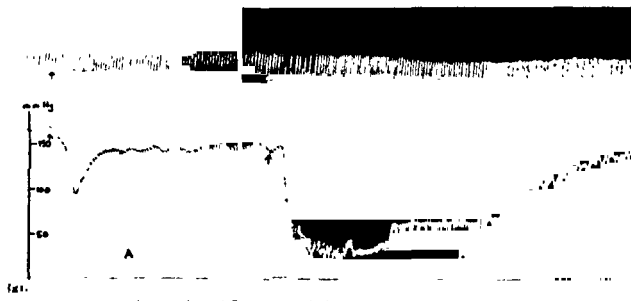


FIG. 4. VERATRIDINE 1 MICROGM./KGM. INJECTED

A. intravenously and B. into left ventricle. Record of respiration, arterial blood pressure, signal, and time interval of 5 seconds. Dog, 14 kgm.

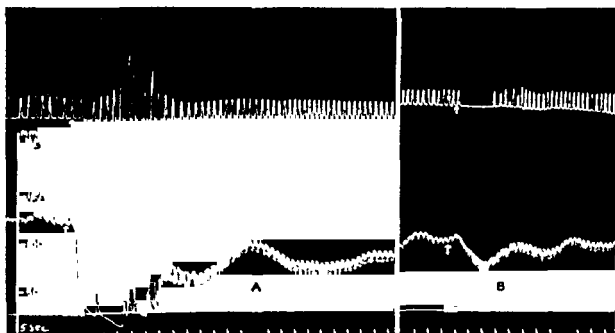


FIG. 5. VERATRIDINE 1 MICROGM./KGM. INJECTED

A. left ventricle and B. right pulmonary artery. Record of respiration, arterial blood pressure, signal, and time interval of 5 seconds. Dog, 17 kgm.

sible for the veratridine apnea while the circulatory effects probably are due to access of the drug to the cardiac receptors described in the preceding section.

Further confirmation of this conclusion was obtained by inhalation of veratridine in aerosol form through the tracheal cannula. This produced distinct

apnea, usually without bradycardia or hypotension (fig. 7). The effect was abolished by block of the two vagus nerves but attempts at anesthetizing the receptors by inhalation of 1 to 5 per cent cocaine hydrochloride solution in aerosol form were unsuccessful. Nevertheless the occurrence of apnea without the usual cardiovascular effects when veratridine was inhaled indicates that at least some

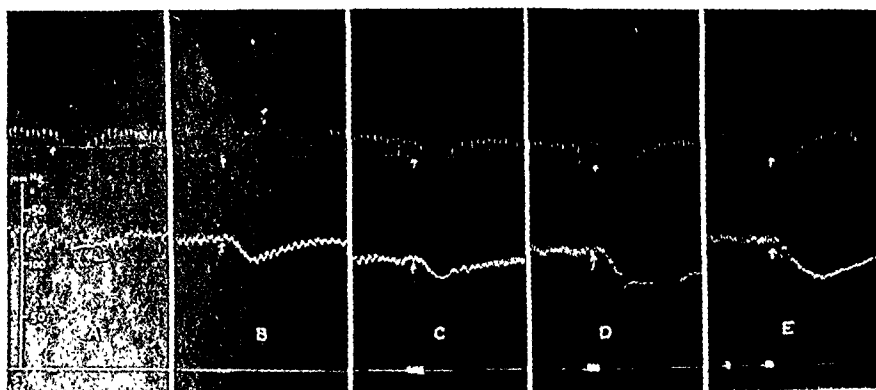


FIG. 6. EFFECTS OF REVERSIBLE BLOCK OF CERVICAL VAGUS ON VERATRIDINE 1 MICROGM./KGM.; INJECTED INTO LEFT PULMONARY ARTERY, UNDER FOLLOWING CONDITIONS:

A. vagi intact; B. cold block of left vagus; C. after its recovery; D. cold block of right vagus; E. after its recovery. Record of respiration, arterial blood pressure, signal, and time interval of 5 seconds. Dog, 17 kgm.

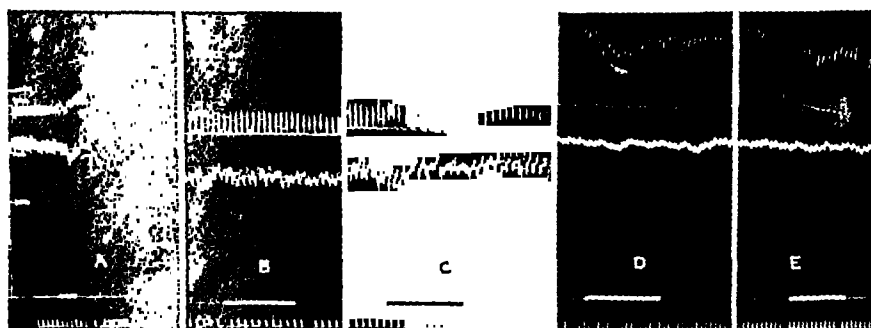


FIG. 7. INHALATION INTO LOWER RESPIRATORY TRACT

A. ether, B. saline, C. veratridine solution, 5 mgm./cc. After cutting vagi, D. saline and E. veratridine inhalations repeated. Record of respiration, arterial blood pressure, signal, and time interval of 5 seconds. Dog, 10.5 kgm.

of the receptors lie in intimate relation with the pulmonary alveoli. Further localization was not attempted.

C. Carotid receptors. Injection of a suitable dose (1 microgm. or more) of veratridine into one common carotid artery by way of its superior thyroid branch led almost immediately to bradycardia and hypotension, but the apnea characteristic of the Bezold effect now was replaced by hyperpnea similar to that elicited by cyanide or lobeline (fig. 8). Exactly the same results were obtained

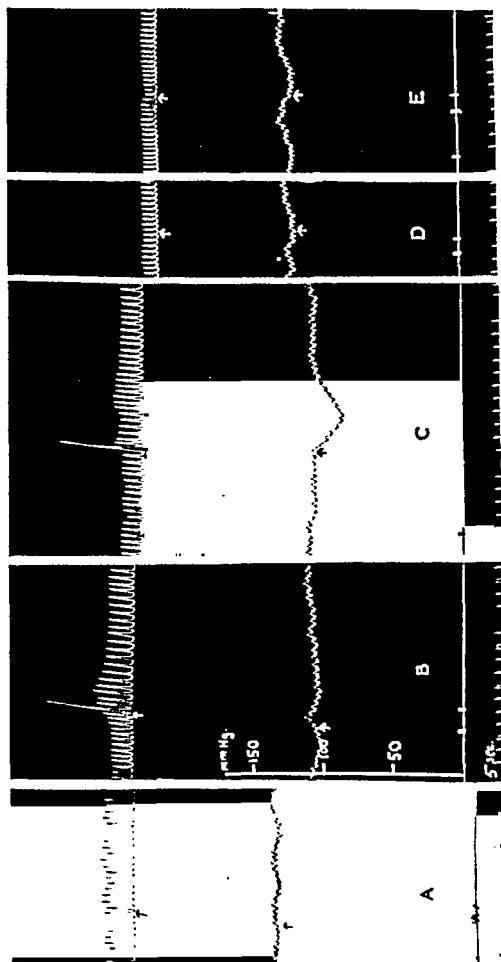


FIG. 8. VERATRIDINE ON CAROTID RECEPTORS

A. veratridine 10 microgm. injected into carotid sinus pressoreceptors, B. sodium cyanide 0.1 mgm. and C. veratridine 2 microgm. injected into isolated carotid body. Repetition of D. sodium cyanide and E. veratridine after cutting sinus nerve. Record of respiration, arterial blood pressure, signal, and time interval of 5 seconds. Dog, 14.2 kgm.

when veratridine was injected into the carotid body region isolated by ligatures from the carotid sinus (fig. 1). Injection into the carotid sinus-internal carotid region separated by ligature from the carotid body (fig. 1) had no effect. Section of the sinus nerve led to complete abolition of bradycardia, hypotension and hyperpnea from any intracarotid injections of veratridine in six of nine dogs tested, whereas hypotension persisted in the other three.

Thus it appears that veratridine is capable of eliciting its characteristic cardiac slowing and hypotension from structures in the carotid body region, but the concomitant respiratory effect in our animals was stimulation, not depression. The effects upon heart rate and respiration appear to be entirely due to reflexes from the carotid body region, since they were abolished by ipsilateral section of the sinus nerve. The same statement can be made about the hypotension in six of the nine animals. Its persistence after denervation in the remaining three probably was not due to an action on the central nervous system since no such effect was seen upon injections of the same doses into the carotid sinus. This hypotension was accompanied by increased blood flow in the hind limb, as measured by a thermistromuhr (15) applied to the femoral vein.

DISCUSSION. The results of our experiments on nearly intact dogs agree with those of Dawes (7) on cats with exteriorized hearts with regard to a separate location of the receptors responsible for the respiratory and cardiovascular effects of veratrum (the Bezold effect). The characteristic respiratory depression or apnea originates in pulmonary receptors which can be activated either by injections of veratridine into the pulmonary artery or by inhalation of the drug in aerosol form, without concomitant circulatory effects. The cardiovascular effects (bradycardia and vasodilation) can be elicited by injections into the coronary arteries with negligible effects on respiration or none at all. We were also able to confirm Jarisch and Richter (16) as to the ability of veratrum to set up typical cardiovascular reflexes from the carotid body region of the dog, but we do not believe that this action plays an important part in the Bezold effect as ordinarily seen. Our reasons are that the latter (a) is not significantly altered by denervation of both carotid reflex zones, (b) is completely abolished by bilateral vagotomy although the carotid innervation is intact, and (c) is not seen when a dose of veratridine, which causes immediate bradycardia and vasodilatation when injected into the aorta proximal to the origin of the coronary arteries, is injected distal to that point.

effects of intracarotid injections of veratridine actually conform with the Bezold pattern. They include bradycardia and vasodilation of the Bezold type, but hyperpnea replaced by hyperventilation, a characteristic of the latter. Hyperpnea and hyperventilation occur together when the carotid chemoreceptors are stimulated, but such phenomena are associated with stimulation of the carotid chemoreceptors, not the carotid body. Denervation of the carotids abolishes the hyperpnea, but in intact dogs the hyperventilation persisted. Yet this effect is not seen when veratridine was injected into the carotid body. As noted

above, the Bezold effect duplicates exactly the result of strong stimulation of the carotid sinus pressoreceptors, either by a rise in intra-carotid pressure (5, 6, 19) or by an electrical current applied to the nerve fibers between the carotid sinus and carotid body (17). Yet the total lack of effect from injections of veratridine so that they reach the carotid sinus without touching the carotid body shows that the carotid pressoreceptors are not involved in the Bezold effect. The best explanation of these findings at present is that the action of veratridine on the carotid reflex mechanism is non-specific, resembling that of potassium ions (20, 21) and affecting the chemoreceptors rather than the pressoreceptors because the latter are nearly insensitive to chemical agents in general. This would explain the hyperpnea and bradycardia. The vasodilatation, however, points to the presence in the external carotid distribution of receptors of a type not previously known to exist here. The fact that section of the sinus nerve does not always abolish this effect indicates that the receptors are not confined to the carotid body and may indeed be in an entirely different location.

As far as veratrum apnea is concerned, we can fully confirm Dawes' (7) conclusion that this is due to reflexes from special receptors somewhere in the lungs. Their exact location is still uncertain but the occurrence of apnea on inhalation of a veratridine aerosol shows that some of these receptors must lie in the alveolar walls or pulmonary capillaries and veins. The absence of concomitant circulatory effects indicates that systemic absorption is not involved. The apnea is due to a specific reflex, not to irradiation into the respiratory center of strong impulses intended primarily for the vasomotor and cardioregulatory center, because the respiratory effect—(a) was minimal or absent when veratridine was injected into the coronary stream and maximum bradycardia and vasodilation were present; (b) began almost instantly on injection of the drug into the pulmonary artery while the circulatory effects were delayed for a period corresponding closely with the pulmonary circulation time, as indicated by similar injections of acetylcholine or cyanide; (c) on injection into one pulmonary artery, was abolished by blocking the ipsilateral vagus while the circulatory effects occurred much as before; and (d) could be elicited by inhalation of veratridine vapor without any cardiovascular effects.

The respiratory effect apparently was entirely reflex, at least in the doses used, because it was abolished by vagus block or section and was not elicited by injections through the internal carotid artery. Intracarotid injections actually caused hyperpnea instead of apnea but the absence of this effect after section of the sinus nerves shows that it was not central in origin.

The physiological significance of the veratrum-sensitive reflex system is as obscure to us as it has been to others (2, 4, 7). The suggestion (7) that the apnea is due to stimulation by veratrum of pulmonary stretch receptors leaves unexplained the lack of involvement of other pulmonary stretch receptors which stimulate breathing (22, 23). One of the most striking attributes of stretch receptors is their insensitivity to chemical agents (24), and our results with injections of veratridine into the carotid sinus indicate that these stretch receptors are indeed insensitive to this drug. The same objection applies to the suggestion (7,

25) that the cardiovascular effects elicited by veratridine are due to an action by the drug on stretch receptors in heart chambers, coronary artery and pulmonary artery. Nevertheless it is difficult to conceive that the reflex system through which the Bezold effect is elicited can be activated only by some drugs to which few if any ancestral forms could have been exposed. Actually a few less exotic substances have been found capable of activating this mechanism. These are foreign serum (26, 27), ethylacetoacetate (28), nicotine (7), adenosine triphosphate (29), and some antihistaminic agents (30). At the present time there is no evident common property in this list and the physiological stimulus to the system is obscure. The main significance of the veratrum alkaloids lies not in their therapeutic value, but in their availability as tools to aid in the identification, localization and eventual elucidation of this extraordinary mechanism.

SUMMARY AND CONCLUSIONS

The mechanism of apnea following an intravenous injection of veratridine was investigated on nearly intact dogs by catheterization of their cardiopulmonary circulation. It was found to be a reflex from special receptors in the lungs that were accessible to the pulmonary circulation and alveolar air.

Injections of veratridine into the coronary arteries caused reflex bradycardia and vasodilatation with negligible effects on respiration. Injections into the carotid body areas initiated similar circulatory changes.

These veratridine-sensitive receptors in the lungs, heart and carotid body area await further investigation as to their physiological significance.

REFERENCES

1. AVIADO, D. M., JR., AND PONTIUS, R. G.: *Fed. Proc.*, **8**: 5, 1949.
2. AVIADO, D. M., JR., AND PONTIUS, R. G.: *Am. J. Med. Sci.*, **218**: 115, 1949.
3. KRAYER, O., AND ACHESON, G. H.: *Physiol. Rev.*, **26**: 383, 1946.
4. BEZOLD, A. VON, AND HIRT, L.: *Unters. Physiol. Lab. Wurzburg*, **1**: 73, 1867.
5. SCHMIDT, C. F.: *Am. J. Physiol.*, **102**: 94, 1932.
6. HEYMANS, C., BOUCKAERT, J. J., AND REGNIERS, P.: *Le Sinus Carotidien et La Zone Homologue Cardio-aortique*, Paris, 1933.
7. DAVES, G. S.: *THIS JOURNAL*, **89**: 325, 1947.
8. COMROE, J. H., JR., AND SCHMIDT, C. F.: *Am. J. Physiol.*, **121**: 75, 1938.
9. MENDEZ, R., AND MONTES, G.: *THIS JOURNAL*, **78**: 238, 1943.
10. MOE, G. K., BASSETT, D. L., AND KRAYER, O.: *THIS JOURNAL*, **80**: 272, 1944.
11. KOCH, E.: *Die Reflektorische Selbststeuerung des Kreislaufes*, Dresden, 1931.
12. CRAMER, W.: *THIS JOURNAL*, **7**: 63, 1915.
13. KRAYER, O., WOOD, E. H., AND MONTES, G.: *THIS JOURNAL*, **79**: 215, 1943.
14. CHEN, K. K., AND SCHMIDT, C. F.: *Medicine*, **9**: 1, 1930.
15. SCHMIDT, C. F., AND WALKER, A. W.: *Proc. Soc. Exper. Biol. and Med.*, **33**: 346, 1935.
16. JARISCH, A., AND RICHTER, H.: *Arch. Exper. Path. u. Pharmacol.*, **193**: 347, 355, 1939.
17. SCHMIDT, C. F.: *Anesth. and Analg.*, **19**: 261, 1940.
18. SCHMIDT, C. F., AND COMROE, J. H., JR.: *Physiol. Rev.*, **20**: 115, 1940.
19. HEYMANS, C., AND BOUCKAERT, J. J.: *J. Physiol.*, **69**: 254, 1930.
20. EULER, U. S. VON: *Skandinav. Arch. f. Physiol.*, **80**: 94, 1938.
21. WINDER, C. V.: *Am. J. Physiol.*, **126**: 655, 1939.

22. HAMMOUDO, M., AND WILSON, W. H.: *J. Physiol.*, 83: 232, 1935; 85: 62, 1935; 94: 497, 1939.
23. LARRABEE, M. G., AND KNOWLTON, G. C.: *Am. J. Physiol.*, 147: 90, 1916.
24. ADRIAN, E. D.: *J. Physiol.*, 79: 332, 1933.
25. JARISCH, A., AND RICHTER, H.: *Klin. Wehnschr.*, 1: 185, 1939.
26. BRODIE, T. G., AND RUSSELL, A. E.: *J. Physiol.*, 26: 92, 1900.
27. PONDER, E.: *Quart. J. Exper. Physiol.*, 18: 133, 1927.
28. BAGOURY, M. M., AND SAMAAH, A.: *J. Egyptian Med. A.*, 24: 211, 1911.
29. EMMELIN, N., AND FELDBERG, W.: *Brit. J. Pharmacol.*, 3: 273, 1948.
30. AVIADO, D. M., JR., AND PONTIUS, R. G.: Unpublished.

THE EFFECTS OF THIOUREAS AND RELATED COMPOUNDS ON ALPHANAPHTHYLTHIOUREA (ANTU) TOXICITY TO RATS¹

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In an effort to elucidate the mechanism of action of the rodenticide alphanaphthylthiourea (ANTU), a thiourea derivative which causes acute pulmonary edema and pleural effusion in susceptible animals without demonstrably affecting extrapulmonary tissues,⁴ this laboratory has studied the effects of a large number of compounds on ANTU toxicity to rats. Our results with the majority of these have previously been reported (1, 2), and may be summarized as follows. Potassium iodide was found to give a high degree of protection provided that it was administered prophylactically more than six hours prior to the injection of two LD₅₀'s of ANTU. Cysteine, when given simultaneously with the rodenticide, delayed the onset of symptoms and time of death, but did not decrease mortality while l-thiosorbitol administered similarly produced a significant decrease in mortality. Neither of these compounds, however, was effective if given as little as $\frac{1}{2}$ hour after the injection of ANTU. On the other hand, n-amyl mercaptan conferred a significant degree of protection against ANTU when the former was given one hour before, simultaneously with, or one hour after the rodenticide; if given two hours after, it no longer reduced mortality but still delayed the onset of symptoms and time of death.

It has previously been suggested (1) that these -SH containing compounds may exert their protective action by competing with ANTU for essential tissue constituents. It occurred to the authors that compounds structurally related to ANTU but having less toxicity might prove more effective in counteracting the lethality of the rodenticide to rats. The present study was undertaken to test this hypothesis.

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⁴ Norway rats, albino rats and mice, pigs and dogs are reported to be the most susceptible animals to ANTU (killed by doses less than 50 mgm./kgm.) (3, 4). Accidental poisonings of cats and dogs have occurred. So far as is known at present ANTU is probably non-toxic to man, except, perhaps, in large amounts. No cases of human poisoning have been reported.

MATERIALS AND METHODS. The compounds tested in this study* included five singly N-substituted thiourea derivatives, eight symmetrically disubstituted thiourea derivatives and three related substances. The singly N-substituted derivatives included three N-alkyl thioureas (allyl, isopropyl and n-lauryl), one N-acylthiourea (acetyl) and one N-aryl thiourea (2,5-dichlorophenyl). The symmetrically disubstituted group contained two N,N'-dialkylthioureas (di-n-butyl and di-isopropyl), three N,N'-diarylthioureas (di-2-chlorophenyl, di-1-naphthyl and di-o-tolyl) and three compounds with the thioureylene grouping as part of a ring (thiouracil, 2-imidazolidinethione [ethylene thiourea] and 5-chloro-2(3)-benzimidazolethione). The remaining compounds included one derivative with a divalent substituent on one nitrogen (ethylidene thiourea) and two thiazoles (2-mercaptobenzothiazole and 2-thioureido-4-methyl thiazole). The formulae for these substances are presented in table 2.

Wistar, albino rats of both sexes, weighing from 220 to 300 gm., were used throughout these experiments. The animals were born and raised in our animal colony and maintained for no less than one month on a diet previously described (1). Injections of ANTU and all compounds tested, with the exception of thiouracil, were given by the intraperitoneal route with constant non-toxic quantities of propylene glycol (1.0 cc./kgm.) as the vehicle. Thiouracil was administered by the same route as a suspension in aqueous 1 per cent sodium methyl carboxylic cellulose. Simultaneous injections of ANTU and test-compounds were given separately into different areas of the abdomen. All compounds were tested for toxicity prior to their use in an experiment; those that were found to be moderately toxic (deaths occurring with doses of 50 mgm./kgm. or less) were administered in maximal sub-lethal dosage.

Tests were conducted in groups of six or ten animals. Control animals were injected on each test-day with the same dose of ANTU (2 LD₅₀'s[†] = 5.0 mgm./kgm.) as given to rats subjected to treatment. Both control and experimental (treated) groups were observed for a period of five days following the injection of ANTU. Autopsies were done whenever there was a question as to the cause of death.

RESULTS. Ethylene thiourea (2-imidazolidinethione) was the first of the group to be found effective in counteracting the toxic effects of ANTU. Because a comparatively large supply of this substance was at hand, in contrast to the small amounts of the other compounds available, more data were assembled for this compound than for any of the others. Ethylene thiourea significantly reduced mortality among ANTU poisoned rats when given simultaneously with two LD₅₀'s of the rodenticide, and this protection was apparently more or less constant, regardless of the dose administered or route of injection (table 1). Because there proved to be no significant difference between the degree of protection furnished by 200 mgm./kgm. and that furnished by 25 mgm./kgm. doses, a 50 mgm./kgm. dose was arbitrarily selected for further studies with this compound.

When this amount of ethylene thiourea was injected simultaneously with two LD₅₀'s of ANTU, the expected mortality was reduced from 89 per cent to 40

* With the exceptions of ethylene thiourea and 2-thioureido-4-methylthiazole, all of the compounds tested were kindly furnished by Doctors Curt P. Richter and Sally H. Dieke, Psychobiological Laboratory, Johns Hopkins Medical School, who, in turn, received them from the Eastman Kodak, Du Pont, Merck and American Cyanamid Companies (5). The ethylene thiourea was obtained through the courtesy of the Rohm and Haas Company, Philadelphia. The thiouracil was kindly furnished by the Lederle Laboratories, Inc.

[†] LD₅₀ = 2.47 mgm./kgm., calculated by the method of Bliss (6).

per cent⁷ and the survival time of treated animals dying was increased (table 2). When given prior to the injection of the rodenticide, ethylene thiourea afforded increasing amounts of protection as the interval between the time of its administration and that of ANTU was increased; i.e.—when injected fifteen minutes before, it decreased mortality from 85 per cent to 35 per cent, one hour before—from 80 per cent to 20 per cent, two hours or 24 hours before—from 70 per cent or 84 per cent to zero (table 3). It is interesting to note, however, that the injection of this compound six hours before decreased the mortality from 70 per cent to 10 per cent, suggesting less protection when given six hours before than when given two hours prior to the injection of ANTU. Although the mortality difference is not statistically significant, it was observed in the two experiments performed that the symptoms manifested by surviving rats (principally dyspnea) were more severe, on the whole, in the six-hour group than in the two-hour group.

TABLE 1

*Effect of various doses of ethylene thiourea when given simultaneously with ANTU**

DOSE	ROUTE†	MORTALITY				APPROXIMATE MEAN SURVIVAL TIME (HOURS) OF ANIMALS DYING	
		Control‡		Experimental		Control	Experimental
		fract.	%	fract.	%		
<i>mgm./kgm.</i>							
200	I.P.	10/10	100	3/10	30 (✓)	14.1	23.6
100	I.P.	10/10	100	5/10	50 (✓)	14.1	20.4
50	I.P.	10/10	100	2/10	20 (✓)	14.1	20.0
50	S.Q.	10/10	100	2/10	20 (✓)	14.1	22.0
25	I.P.	10/10	100	4/10	40 (✓)	14.1	21.8

* 2 LD₅₀'s (5.0 mgm./kgm.), equivalent to an expected mortality of ca. 90 per cent.

† Route of injection, I.P. = intraperitoneally, S.Q. = subcutaneously.

‡ These experiments were run on the same day; one control group, injected with ANTU only, served for all.

(✓) $P < 0.01$ by Student's "t" Test (13).

When the ethylene thiourea was administered one or two hours after the injection of ANTU it still afforded apparent protection, decreasing mortality from 80 per cent and 70 per cent to 57 per cent and 40 per cent, respectively, and increasing survival time (table 4).

It is well known that Norway rats rapidly develop tolerance to ANTU (7-11) and that a small dose of the rodenticide administered a sufficient period of time beforehand will protect against an otherwise lethal dose given later.⁸ In order to

⁷ The expression "mortality reduced from 89 per cent to 40 per cent" as used here and throughout the paper will indicate that the mortality was reduced from a figure of 89 per cent in the control group to a figure of 40 per cent in the experimental group.

⁸ In order to determine whether or not there was some substance present in the blood of rats made tolerant to ANTU which served to protect them from the rodenticide, the following experiments were carried out.

1. A group of five rats was injected intraperitoneally with the serum (1.5 cc. per rat) of animals that had built up a tolerance to more than fifteen LD₅₀'s of ANTU, simultaneously

TABLE 2
Effect of thioureas and related compounds when given simultaneously with ANTU*

COMPOUND	FORMULA	DOSE† mgm./ kgm.	MORTALITY				APPROXIMATE MEAN SURVIVAL TIME (HOURS) OF ANIMALS DYING	
			Control		Experimental		Control	Experimental
			fract.	%	fract.	%		
Acetyl thiourea	$\text{CH}_3\text{CO}\cdot\text{NHCSNH}_2$	20	5/6	83	5/6	83	13.6	13.0
Allyl thiourea	$\text{CH}_2=\text{CHCH}_2\cdot\text{NHCSNH}_2$	50	18/20	90	2/16	12.5 (✓)	11.7	7.5
Isopropyl thiourea	$(\text{CH}_3)_2\text{CH}\cdot\text{NHCSNH}_2$	100	10/12	83	3/12	25 (✓)	12.5	3.7
		50	13/16	81	0/16	0 (✓)	13.0	—
n-Lauryl thiourea	$\text{CH}_3(\text{CH}_2)_{11}\cdot\text{NHCSNH}_2$	25	5/6	83	6/6	100	12.0	4.7
2,5-Dichlorophenyl thiourea	$\text{Cl}_2\text{C}_6\text{H}_3\cdot\text{NHCSNH}_2$	100	5/6	83	5/6	83	13.6	12.0
		50	17/20	85	13/16	81	12.3	20.5
Di-n-butyl thiourea	$\text{CH}_3(\text{CH}_2)_3\cdot\text{NHCSNH}_2$	100	5/6	83	4/6	67	13.6	4.5
		50	5/6	83	6/6	100	12.0	7.7
Di-isopropyl thiourea	$(\text{CH}_3)_2\text{CH}\cdot\text{NHCSNH}_2$	75	9/10	90	10/10	100	12.0	12.0
Di-2-chlorophenyl thiourea	$\text{Cl}_2\text{C}_6\text{H}_3\cdot\text{NHCSNH}_2$	100	8/10	80	6/6	100	10.9	12.0
Di-1-naphthyl thiourea	$\text{C}_{10}\text{H}_7\cdot\text{NHCSNH}_2$	100	5/6	83	6/6	100	13.0	9.2
Di-o-tolyl thiourea	$\text{CH}_3\text{C}_6\text{H}_4\cdot\text{NHCSNH}_2$	100	5/6	83	6/6	100	13.6	10.5
Thiouracil	$\text{CH}_3\cdot\text{CH}\cdot\text{NHCSNH}_2\cdot\text{CO}$	75	8/10	80	10/10	100	10.9	11.2
2-Imidazolidinethione (ethylene thiourea)	$\text{CH}_2\cdot\text{NHCSNH}_2\cdot\text{CH}_2$	50	41/46	89	23/38	40 (✓)	11.8	16.8
5-Chloro-2(3)-benzimidazolethione	$\text{ClC}_6\text{H}_4\cdot\text{NHCSNH}_2$	50	10/12	83	8/12	67	12.8	16.6
Ethylidene thiourea	$\text{CH}_3\text{CH}=\text{NCSNH}_2$	25	22/26	85	11/26	12 (✓)	12.2	14.6
		50	5/6	83	4/6	67	13.6	9.8
2-Mercapto-benzothiazole	$\text{C}_6\text{H}_4\text{SC(SH)}\cdot\text{N}$	25	27/32	84	16/28	57†	12.3	11.3
2-Thioureido-4-methylthiazole	$\text{CH}_3\text{C}=\text{CHSC}(\text{NHCSNH}_2)_2\cdot\text{N}$	100	10/12	83	10/12	83	12.8	9.5
		50	5/6	83	6/6	100	13.6	12.0

* $\text{C}_6\text{H}_5\cdot\text{NHCSNH}_2$, injected intraperitoneally in a dose of two LD_{50} 's (5.0 mgm./kgm.), equivalent to an expected mortality of ca. 90 per cent.

† Injected intraperitoneally in a constant, non-toxic quantity of propylene glycol (1.0 cc./kgm.).

(✓) $P < 0.01$ by Student's "t" Test (13).

‡ $P = 0.018$.

compare the protective effect of prophylactically administered ANTU with that of prophylactically administered ethylene thiourea, an experiment was planned in which several groups of rats received either ethylene thiourea or a small dose of ANTU at varying lengths of time prior to the administration of two LD₅₀'s of the rodenticide. The results are summarized in table 3. When ANTU was injected in a dose of 1 mgm./kgm., it killed one of six animals; the remaining five however were protected against two LD₅₀'s administered 24 hours later. Two of the five rats were moderately dyspneic but recovered. A dose of 0.5 mgm./kgm. killed none of six animals; however, two of the six died when injected with two LD₅₀'s 24 hours later. Survival time was greater than that of the controls. When ANTU

TABLE 3

*Effect of thiourcas and related compounds when given prior to ANTU**

COMPOUND	DOSE†	TIME OF INJECTION (HOURS BEFORE ANTU)	MORTALITY				APPROXIMATE MEAN SURVIVAL TIME (HOURS) OF ANIMALS DYING	
			Control		Experimental			
			fract.	%	fract.	%	Control	Experi- mental
	mgm./ kgm.							
Isopropyl Thiourea	50	1	8/10	80	0/10	0 (✓)	13.0	—
Ethylene Thiourea	50	.25	22/26	85	9/26	35 (✓)	13.7	19.0
Ethylene Thiourea	50	1	16/20	80	4/20	20 (✓)	11.5	22.8
Ethylene Thiourea	50	2	14/20	70	0/20	0 (✓)	13.0	—
Ethylene Thiourea	50	6	14/20	70	2/20	10 (✓)	13.0	13.0
Ethylene Thiourea	50	24	30/36	83	0/28	0 (✓)	12.0	—
ANTU	0.5	2	8/10	80	10/10	100	13.0	13.0
ANTU	0.5	6	8/10	80	9/10	90	13.0	14.1
ANTU	0.5	24	8/10	80	2/6	33	13.0	17.5
ANTU	1.0	24	8/10	80	0/5‡	0 (✓)	13.0	—

* Injected intraperitoneally in a dose of two LD₅₀'s (5.0 mgm./kgm.), equivalent to an expected mortality of ca. 90 per cent.

† Injected intraperitoneally in a constant, non-toxic quantity of propylene glycol (1.0 cc./kgm.).

‡ There were originally six animals in this group, but one died with symptoms of ANTU poisoning following the 1.0 mgm./kgm. dose.

(✓) $P < 0.01$ by Student's "t" Test (13).

was administered in a dose of 0.5 mgm./kgm. either six or two hours prior to the injection of two LD₅₀'s, it completely failed to protect, more rats dying in the experimental than in the control groups. Ethylene thiourea, on the other hand, afforded as much protection when it was administered two hours before the rodenticide as it did when given 24 hours before.

with the administration of two LD₅₀'s of the poison. All of the animals died. Rats receiving the serum alone showed no ill effects.

2. A group of 25 rats received two LD₅₀'s of ANTU after the solution of the latter had been mixed (1.5:1) with the serum of rats tolerant to more than fifteen LD₅₀'s. Injections of the mixture either immediately after mixing or following a five-hour incubation period at 37°C. were equally lethal; 23 of the animals died and no increase in survival time was observed.

Of the remainder of the thioureas and related compounds tested, only two, the isopropyl and allyl thioureas, offered highly significant protection against ANTU (table 2). 5-Chloro-2(3)-benzimidazolethione conferred less, but still definitely significant, protection, while ethylidene thiourea offered protection which was probably significant. 2,5-Dichlorophenyl thiourea did not decrease mortality but produced a markedly increased average survival time. All of the remaining compounds were completely ineffective in counteracting the toxic effects of ANTU.

The isopropyl and allyl thioureas turned out to be the most effective therapeutic agents against ANTU that we have so far discovered. When a 100 mgm./kgm. dose of isopropyl thiourea was injected at the same time as two LD₅₀'s of ANTU, it decreased the mortality from 83 per cent to 25 per cent, and when given in a dose of 50 mgm./kgm. it reduced the mortality to zero. Although a few of these rats became moderately dyspneic, all recovered. As will be mentioned

TABLE 4
*Effect of thioureas and related compounds when given after ANTU**

COMPOUND	DOSE†	TIME OF INJECTION (HOURS AFTER ANTU)	MORTALITY				APPROXIMATE MEAN SURVIVAL TIME (HOURS) OF ANIMALS DYING	
			Control		Experimental		Control	Experimental
			fract.	%	fract.	%		
Isopropyl Thiourea	50	1	8/10	80	1/10	10 (✓)	13.0	30.0
Allyl Thiourea	50	1	8/10	80	0/10	0 (✓)	10.9	—
Ethylene Thiourea	50	1	24/30	80	17/30	57	12.0	16.5
Ethylene Thiourea	50	2	14/20	70	8/20	40	13.0	15.7

* Injected intraperitoneally in a dose of two LD₅₀'s (5.0 mgm./kgm.), equivalent to an expected mortality of ca. 90 per cent.

† Injected intraperitoneally in a constant, non-toxic quantity of propylene glycol (1.0 cc./kgm.).

(✓) $P < 0.01$ by Student's "t" Test (13).

again later on, most of the thiourea compounds, when given in sufficient amounts, produce the same symptoms and pathological findings, and probably kill by the same mechanism, as does ANTU (5). The 100 mgm./kgm. dose of isopropyl thiourea is one-fifth of its approximate LD₅₀, as determined by Dieke (5), and may have been too large to exert an optimum amount of protective action. At any rate, the smaller dose of the compound was more effective; the same type of thing seems to have occurred with 2,5-dichlorophenyl thiourea, 5-chloro-2(3)-benzimidazolethione and ethylidene thiourea (see below). Isopropyl thiourea also decreased the mortality to zero when 50 mgm./kgm. were administered one hour before the rodenticide (table 3), the rats showing slight, if any, symptoms of poisoning. When given one hour after ANTU, this thiourea still exerted a marked degree of protection, only one of ten animals dying (table 4).

Allyl thiourea, administered in a dose of 50 mgm./kgm. simultaneously with ANTU, decreased the number of deaths from the expected 90 per cent to 15 per

cent (table 2). When this amount was given one hour after the injection of ANTU, it completely protected the rats from the lethal effects of the rodenticide (table 3). Several of the animals became severely dyspneic, but all recovered. Neither 5-chloro-2(3)-benzimidazoethione nor ethylidene thiourea conferred a significant amount of protection when administered in a dose of 50 mgm./kgm. However, when this dose was halved to 25 mgm./kgm., mortality was reduced from 85 per cent and 84 per cent to 42 per cent and 57 per cent, respectively (table 2).

It should be noted from table 2 that while the isopropyl and allyl thioureas were highly effective in protecting rats against the lethal effects of ANTU, the average survival time of the treated animals that did die was markedly shorter than that of the control animals. This acceleration of the appearance of symptoms and the time of death of certain individuals was caused by ethylidene thiourea also, as well as by many of the compounds that failed to protect, especially among the symmetrically disubstituted thiourea derivatives. The action of 5-chloro-2(3)-benzimidazoethione, however, resembled that of ethylene thiourea not only in the degree of protection conferred, but also in that it usually produced a definite delay in the onset of symptoms and time of death⁹. Both are compounds having the thioureylene grouping as part of a ring.

DISCUSSION. An examination of the structures of the compounds found to give prophylactic and/or antidotal protection against ANTU poisoning in rats shows that they can be classified into two groups: (a) alkyl-monosubstituted thioureas and (b) compounds with the thioureylene group as part of the ring. Two compounds in the first group were found to be effective: isopropyl thiourea and allyl thiourea. While allyl thiourea seems to be the most promising compound since it decreased mortality from 80 per cent to 0 per cent when given one hour after ANTU, the degree of protection offered by it was not significantly different from that offered by isopropyl thiourea. Ethylidene thiourea, a thiourea with a divalent substituent on one nitrogen and thus a compound closely related to the alkyl-monosubstituted thioureas, offered probably significant protection when given simultaneously with ANTU. The two compounds found effective in group (b) were ethylene thiourea and 5-chloro-2(3)-benzimidazoethione. Thiouracil, also a member of this group, was totally ineffective when given simultaneously with ANTU. However, this compound is very insoluble and enough may not have been absorbed in time to be effective. The compounds of group (a) differed markedly from those of group (b) in that the survival time of rats dying was decreased by the former. This property was shared by the ineffective compounds of group (a) as well as by the alkyl- and aryl-disubstituted thioureas that we have tested. However, the aryl-monosubstituted thiourea, 2,5-dichlorophenyl thiourea, when given in a dose of 50 mgm./kgm. increased the mean survival time from twelve to twenty hours without decreasing mortality. Thus the substitution of chlorine in the 2 and 5 positions of the benzene ring not only greatly decreases the toxicity of phenyl thiourea and 2-chlorophenyl thiourea (5), but apparently provides a small degree of ANTU-antagonistic action.

⁹ This compound, when administered in a dose of 50 mgm./kgm. simultaneously with ANTU, caused nearly complete prostration for 24 hours or more. The 25 mgm./kgm. dose caused semi-prostration for a period of several hours.

We have observed that lethal quantities of allyl, n-lauryl, di-isopropyl and ethylidene thiourea will all produce pulmonary edema and pleural effusion, indicating, in all probability, a mechanism of action similar to that of ANTU itself. While limited supply prevented us from administering the other compounds in lethal amounts, Dieke and her coworkers (5) have shown that a large number of the thiourea derivatives they screened for toxicity to rats shared the capacity to cause increased capillary permeability in the lungs. These included methyl, acetyl, allyl and, probably, isopropyl thioureas, as well as a large number of N-aryl derivatives. DuBois *et al.* (14) have reported that a toxic dose of allyl thiourea has the same effect as does ANTU on the blood sugar and liver glycogen of the rat, producing a rapidly appearing hyperglycemia and a marked persistent drop in liver glycogen. It seems probable, therefore, that thiourea derivatives confer antidotal protection by acting as competitive antagonists against ANTU. Elvehjem has recently briefly reviewed the subject of metabolic antagonists (15) and has indicated that it is now generally recognized that slight modifications in the structure of certain vitamins, for example, may change them from essential nutrients to metabolic antagonists, as illustrated by the well-known anti-para-aminobenzoic acid activity of sulfanilamide. Similarly, a modification of the structure of ANTU apparently enables the altered compound to counteract the toxic effects of ANTU in the animal body.

Structural differences, as might be expected, apparently govern the protective action of a given thiourea. Thus n-lauryl, the long chain alkyl-monosubstituted thiourea, was ineffective, whereas the short chain alkyl-monosubstituted thioureas, isopropyl and allyl, were highly protective. Further studies are now in progress to examine a more complete series of monosubstituted thioureas.

SUMMARY

1. Two alkyl singly N-substituted thiourea derivatives, allyl thiourea and isopropyl thiourea, administered in an intraperitoneal dose of 50 mgm./kgm. were effective in significantly reducing mortality in rats when given simultaneously with or one hour after two LD₅₀'s (5 mgm./kgm.) of ANTU. All the compounds of this type tested, as well as several less effective alkyl- and aryl-disubstituted thioureas, reduced survival time of rats dying.

2. Ethylidene thiourea, a thiourea with a divalent substituent on one nitrogen, was also effective in reducing mortality when given in a dose of 25 mgm./kgm. simultaneously with ANTU.

3. Two compounds with the thioureylene grouping as part of a ring, ethylene thiourea and 5-chloro-2(3)-benzimidazolethione, significantly reduced mortality when given simultaneously with ANTU. Ethylene thiourea also significantly reduced mortality when given one, two, six and 24 hours before ANTU and afforded apparent protection when administered one or two hours after the rodenticide. Both of these compounds increased the survival time of rats dying.

4. It is suggested that these thiourea derivatives confer their protection against ANTU poisoning by acting as competitive antagonists to the rodenticide by virtue of their related structure.

REFERENCES

1. MEYER, B. J., AND KAREL, L.: THIS JOURNAL, 92: 15, 1948.
2. KAREL, L., AND MEYER, B. J.: THIS JOURNAL, 93: 414, 1948.
3. DIEKE, S. H., AND RICHTER, C. P.: Proc. Soc. Exper. Biol. & Med., 62: 22, 1946.
4. ANDERSON, W., AND RICHTER, C. P.: Vet. Med., 41: 302, 1946.
5. DIEKE, S. H., ALLEN, G. S., AND RICHTER, C. P.: THIS JOURNAL, 90: 260, 1947.
6. BLISS, C. I.: Quart. J. Pharm. Pharmacol., 11: 192, 1938.
7. RICHTER, C. P., AND CLISBY, K. H.: Proc. Soc. Exper. Biol. & Med., 48: 684, 1941.
8. RICHTER, C. P., AND CLISBY, K. H.: Arch. Path., 33: 46, 1942.
9. McCLOSKEY, W. T., AND SMITH, M. I.: Public Health Reports, 60: 1101, 1945.
10. RICHTER, C. P.: J. A. M. A., 129: 927, 1945.
11. LANDGREBE, F. W.: Nature, 157: 22, 1946.
12. RICHTER, C. P.: Proc. Soc. Exper. Biol. & Med., 63: 364, 1946.
13. LUYKX, H. M. C.: U. S. Naval Med. Bull., 43: 1208, 1944.
14. DU BOIS, K. P., HERRMANN, R. G., AND ERWAY, W. F.: THIS JOURNAL, 89: 186, 1947.
15. ELVEHJEM, C. A.: J. A. M. A., 136: 915, 1948.

ADRENERGIC BLOCKING DRUGS

V. BLOCKING OF EXCITATORY RESPONSES TO EPINEPHRINE AND ADRENERGIC NERVE STIMULATION WITH N-ALKYL-N-(2-CHLOROETHYL)-BENZHYDRYLAMINES¹

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Pharmacological studies have been made on compounds in a series of benzhydrylamines (1) which contain a 2-chloroethyl group since these compounds are chemically related to N-(2-chloroethyl) dibenzylamine-HCl (Dibenamine) which exerts adrenergic blocking action (2), and are also related to benzhydryl ethers such as diphenhydramine-HCl (Benadryl) which are effective antihistamine compounds (3). We have previously presented evidence that certain 2-chloroethyl derivatives exert a dual block and thereby prevent certain effects of both epinephrine and histamine (4-9). Compounds were therefore examined for adrenergic blocking and antihistamine properties, and some which exerted appreciable adrenergic blocking action were studied in more detail.

METHOD AND RESULTS. The initial studies consisted of screening procedures for presumptive evidence of adrenergic blocking action as indicated by ability of test compounds to diminish the toxicity of epinephrine in mice, and of antihistamine action as revealed by diminution of histamine-induced bronchioconstriction in guinea pigs. Acute oral toxicity was determined in mice so as to permit comparisons of the LD₅₀ with doses used to demonstrate pharmacological activity. Finally, selected compounds were used in dogs and cats to gain information from responses of arterial blood pressure and reactions of the nictitating membrane which concerned adrenergic blocking, antihistamine and atropine-like action.

A. Acute oral toxicity and diminution of epinephrine toxicity in mice: Included in table 1 are the calculated doses of the various compounds which, when administered orally to groups of twenty mice two hours previous to intraperitoneal injection of epinephrine, reduced mortality from 67.0 ± 1.53 per cent (established in 40 groups of twenty, saline-treated control mice) to 33.5 per cent. Details of the procedure and comparisons of several types of adrenergic blocking and other types of drugs have been published (7-9).

It is of interest to note that the methyl homologue (No. 1 in table 1) failed to diminish epinephrine toxicity in mice and that an analogous compound in a series of N-(2-haloalkyl)-1-naphthalenemethylamines (8) exerted less activity than higher homologues. Definite activity was exhibited by ethyl, propyl and butyl homologues (compounds 2 to 7). As indicated previously (7, 8), antagonism of

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epinephrine could be demonstrated one hour following their administration, indicating a more rapid onset of action and/or absorption than with the drug, Dibenamine, which exhibited activity after two or three hours.

A lower degree of activity was demonstrated with higher alkyl homologues (Nos. 7 to 9; isobutyl, n-amyl and n-hexyl). The low solubility of these compounds did not permit preparation of 1.0 per cent aqueous solutions so suspensions in gum acacia were employed. It is improbable that the low activity exhibited was solely due to poor solubility since these three compounds were readily dissolved in propylene glycol, and even then, in doses of 6.0 mgm./kgm., failed to reduce epinephrine toxicity. It is probable that activity was somewhat less in the case of these higher alkyl homologues, as was true in the series of 1-naphthalene-methylamines (8), but which contrasts with the equal activity of an entire series of 2-(2-biphenyloxy)-2'-chlorodiethylamines (9) when administered orally in mice.

Activity was only moderate in the case of the methoxyethyl (No. 10) and 2-amyl (No. 12) derivatives. Substitution of 2-chloropropyl for the 2-chloroethyl group (compare No. 13 and 4) halved the activity. No activity was demonstrated with the secondary 2-chloroethylamine (No. 14) or when a methyl group was substituted for 2-chloroethyl (No. 15).

The acute oral toxicity was determined as previously described (7) and was low with all those compounds examined. Since activity was demonstrated with small doses there is a large ratio of acute toxic dose to the dose employed to demonstrate pharmacological activity in mice.

B. Effect on histamine-induced bronchospasm in guinea pigs: Subcutaneous injections of several compounds were made in groups of twelve guinea pigs 30 minutes before subjecting them to an histamine-aerosol which induced 92 per cent mortality in a like number of controls. Doses of 12.5 and 25.0 mgm./kgm. (Nos. 1, 2, 3, 4, 6, in table 1) failed to diminish bronchospasm sufficiently to reduce mortality, indicating that no appreciable antihistamine activity was demonstrated under the specified conditions. Further search for antihistamine action was made in studies related to vascular responses.

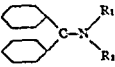
C. Vascular responses to epinephrine, histamine and acetylcholine: Compounds numbered 1 to 6 in the series (table 1) were injected intravenously into one or more intact dogs anesthetized with pentobarbital sodium to determine whether they altered pressor responses to epinephrine hydrochloride (10 microgm.) and depressor responses to histamine diphosphate (50 microgm.) and acetylcholine bromide (25 microgm.). Before the slow intravenous injection (two min.) of the compound being tested, each pressor and depressor agent was injected intravenously twice and such injections were repeated during the intervals 10 to 30, 60 to 90 and 120 to 150 minutes after the test compound.

In each of two experiments, the methyl homologue (No. 1, table 1) in doses of 10.0 mgm./kgm. failed to alter pressor or depressor responses to epinephrine, histamine and acetylcholine; failure to demonstrate epinephrine antagonism thus agrees with the inactivity demonstrated in mice. The ethyl homologue (No. 2 or SY-2), in doses of 3.0, 5.0 and 10.0 mgm./kgm., induced diminution, blockade and reversal of the pressor response to epinephrine, but no impressive

diminution in the depressor responses to histamine and acetylcholine. Similar findings were obtained with the isopropyl homologue (No. 4) and the magnitude of its effect appeared to be comparable to that of the ethyl and isopropyl compounds.

Several other members of the series (Nos. 3, 5 and 6) were less soluble in water so 0.5 per cent solutions were prepared with the aid of propylene glycol (15 per cent final concentration) and injected intravenously. In doses of 10.0 mgm./kgm.,

TABLE I
The antagonism of epinephrine with N-alkyl-N-(2-chloroethyl) benzhydrylamines

Comp. No.*			REDUCTION OF EPINEPHRINE TOXICITY IN MICE EFFECTIVE ORAL DOSE \pm S. E.†	ACUTE TOX- ICITY ORAL, MICE LD ₅₀ \pm S. E.	LD ₅₀ / EFFEC- TIVE DOSE	DOSES WHICH FAILED TO DIMINISH HISTA- MINE-INDUCED BRONCHOSPASM IN GUINEA PIGS (SUBCUTA- NEOUS)
	R ₁	R ₂	mgm./kgm.	mgm./kgm.		mgm./kgm.
1	Methyl	2-chloroethyl	Ineffective at 100			12.5
2 (SY-2)	Ethyl	2-chloroethyl	7.5 \pm 1.1	977 \pm 63	130	12.5
3	n-Propyl	2-chloroethyl	4.9 \pm 0.3	>2000	>480	25.0
4	Isopropyl	2-chloroethyl	3.3 \pm 0.4	1412 \pm 102	427	25.0
5	n-Butyl	2-chloroethyl	7.8 \pm 1.0	>2000	>250	
6	sec.-Butyl	2-chloroethyl	3.4 \pm 0.4	>2000	>558	12.5
7	Isobutyl	2-chloroethyl	33.0 \pm 4.2‡	>2000	>60	
8	n-Amyl	2-chloroethyl	35.0 \pm 5.6‡	>2000	>57	
9	n-Hexyl	2-chloroethyl	21.5 \pm 3.7‡			
10	Methoxyethyl	2-chloroethyl	17.5 \pm 1.4‡			
11	Methallyl	2-chloroethyl	8.6 \pm 1.0			
12	2-Amyl	2-chloroethyl	11.6 \pm 1.4			
13	Isopropyl	2-chloropropyl	7.1 \pm 0.9	1361 \pm 39	19	
14	H	2-chloroethyl	Ineffective at 100			
15	Isopropyl	Methyl	Ineffective at 50			

* The hydrochloride salt was used in all instances.

† Log dose-probit charts used to estimate dose of drug required to halve the mortality in groups of 20 mice receiving epinephrine hydrochloride intraperitoneally (14.4 mgm./kgm.). This dose of epinephrine proved fatal to 67.0 \pm 1.53 per cent of 40 groups of 20 saline-treated, control mice (for details see ref. 7).

‡ Oral administration of suspension in 2.0 per cent gum acacia; when dissolved in propylene glycol these compounds were ineffective at a dose of 6.0 mgm./kgm.

these compounds failed to alter pressor responses to epinephrine and depressor responses to histamine and acetylcholine at any time over a period of two hours. Thus, in the dog these compounds did not exhibit the activity demonstrated with the more soluble ethyl and isopropyl homologues, and under the experimental conditions there was poor correlation between epinephrine antagonism in mice and dogs. Low acute toxicity was revealed for the more insoluble compounds when administered orally to mice.

The ethyl homologue was chosen for further study and comparison with Diben-

amine because its activity appeared to be as great as that exhibited by any other compound in the series and because it was desirable to compare it with ethyl homologues in other series of compounds (7-11). Table 2 contains the means of the several averaged paired responses to epinephrine, histamine and acetylcholine occurring before treatment, and the differences after treatment which indicate the degree of antagonism exerted by SY-2 and Dibenamine.

TABLE 2

Effect of N-ethyl-N-(2-chloroethyl) benzhydrylamine HCl (SY-2) and N-(2-chloroethyl) dibenzylamine HCl (Dibenamine) on pressor responses to epinephrine and depressor responses to histamine and acetylcholine in dogs

COMP. NO.	DOSE I. V.	NO. EXPTS.	CONTROL MEAN PRESSOR RESPONSE \pm S.D.*	AFTER TREATMENT, MEAN DIFFERENCE \pm S.E.†			
				10-30 min.	P	120-150 min.‡	P
Epinephrine Hydrochloride 10 Microgm.							
	mgm./kgm.		mm. Hg	mm. Hg		mm. Hg	
SY-2	5	3	40.0 \pm 6.9	51.1 \pm 5.7	0.02	58.6 \pm 14.7	0.07
SY-2	10	5	35.8 \pm 14.7	60.6 \pm 7.8	0.01	55.4 \pm 9.4	0.01
Dibenamine	10	5	38.2 \pm 23.4	56.0 \pm 12.5	0.01	71.6 \pm 10.7	<0.01
Histamine Diphosphate 50 Microgm.							
			mm. Hg	mm. Hg		mm. Hg	
SY-2	5	3	50.0 \pm 10.0	9.6 \pm 4.2	0.2	5.0 \pm 6.4	0.5
SY-2	10	5	42.0 \pm 11.9	10.8 \pm 1.8	0.01	12.0 \pm 3.2	0.02
Dibenamine	10	5	47.4 \pm 18.3	12.8 \pm 5.2	0.1	20.2 \pm 7.6	0.06
Acetylcholine Bromide 25 Microgm.							
			mm. Hg	mm. Hg		mm. Hg	
SY-2	5	3	44.0 \pm 14.4	5.0 \pm 12.3	0.7	1.7 \pm 5.4	0.8
SY-2	10	5	44.8 \pm 19.0	11.2 \pm 6.3	0.2	16.2 \pm 12.2	0.3
Dibenamine	10	5	39.2 \pm 12.7	3.0 \pm 3.0	0.4	9.6 \pm 4.9	0.2

* Control responses and standard deviations are included to indicate the magnitude and variation of the control observations.

† Statistical evaluation was made by the method of paired data comparisons. Mean differences greater than the control pressor responses indicate epinephrine reversal, i.e., a depressor response. After treatment with each drug, epinephrine usually elicited a pressor spike ranging from 5 to 30 mm. Hg which preceded the depressor response.

‡ Data related to responses at the 60 to 90 minute interval are not recorded because of close similarity to those recorded earlier and later.

In a dose of 3.0 mgm./kgm., SY-2 diminished but did not block responses to epinephrine (unpublished data). In doses of 5.0 and 10.0 mgm./kgm., SY-2 induced epinephrine reversal within ten minutes and such effects persisted throughout the 150-minute period of experimentation. Similar results were obtained with Dibenamine in a dose of 10.0 mgm./kgm. It is apparent that the ethyl homologue in this series of benzhydryl amines is definitely less active as an

adrenergic blocking compound than the ethyl homologues in other series such as N-(2-haloalkyl)-1-naphthalenemethylamines (7, 8, 10), 2-(2-biphenyloxy)-2'-chlorodiethylamines (9), and N-alkyl-N-(2-chloroethyl)-9-fluorenamines (11, 12).

Although SY-2 and several homologues failed to prevent histamine-induced bronchospasm in guinea pigs (table 1), SY-2 in the dose of 10.0 mgm./kgm. did diminish the depressor response to histamine (table 2) in the dog to a small degree. Diminution in response to histamine was about the same in magnitude after Dibenamine, but appeared inconsistently and mean differences did not vary significantly in the experiments made, although data collected at the 120- to 150-minute interval are highly suggestive of an antagonism. Collectively, the data from guinea pigs and dogs do not reveal evidence of any marked degree of histamine antagonism such as that demonstrated with some other halogenated ethylamines which we have studied (4-6, 8, 9). Depressor responses to acetylcholine were not appreciably altered. Therefore, the evidence now available indicates that SY-2 and Dibenamine are fairly specific adrenergic blocking drugs.

D. Adrenergic blocking action in cats: In eight cats anesthetized with pentobarbital sodium, experiments were made to determine the effect of SY-2 on responses of the carotid blood pressure and nictitating membrane to intravenous epinephrine injections, and responses of the nictitating membrane to faradic stimulation of the preganglionic cervical sympathetic nerves. Figure 1 is a portion of a typical experiment. Before intravenous injection of SY-2 (10.0 mgm./kgm.) during a period of two minutes, the mean of sixteen pressor responses to epinephrine (10 microgm.) was 58 mm. Hg. Mean pressor responses to epinephrine were reduced to 18 mm. Hg within ten minutes, blocked at 60 minutes and reversed to a mean depressor response of -15 mm. Hg at 120 minutes. Thus, the dose of SY-2 used in cats did not exert its full epinephrine reversal effects until 120 minutes or later, whereas in the dog, epinephrine reversal appeared to be maximal or nearly so at ten minutes.

The response of the nictitating membrane of cats to the same injections of epinephrine was reduced 75 per cent at 10, 30 and 60 minutes, and almost completely blocked at 120 minutes. Responses of the nictitating membrane following electrical stimulation of the cervical sympathetics for five seconds with a moderate strength of stimulus were reduced by 50 per cent within ten minutes after injection of SY-2, but not reduced more than 75 per cent even after 30, 60 and 120 minutes. In cats, the combined effects of the anesthetic and of SY-2 lowered carotid blood pressure approximately 25 per cent within ten minutes and a major portion of the depression persisted throughout the experimental period. The diminished pressor and nictitating membrane responses recorded after treatment with SY-2 were not due to deterioration of the animal or tissues because in three control experiments the responses being studied were reproduced fully during 120 minutes even though the blood pressure decreased 25 per cent, presumably as a result of the anesthetic.

The fact that SY-2 diminished and finally blocked the response of the nictitating membrane to injected epinephrine again suggests that the drug blocks at the effector cells. Further evidence of peripheral blockade was adduced by

demonstrating that SY-2 greatly diminished the vasoconstriction and pressor responses following direct stimulation of the splanchnic nerves in adrenal-inactivated dogs.

E. Adrenergic blockade in adrenal-inactivated dogs: In three dogs anesthetized with pentobarbital sodium release of epinephrine from the adrenal medulla was prevented by ligation of the adrenal veins. Procedures carried out in duplicate once before and at several time intervals after administration of SY-2 included

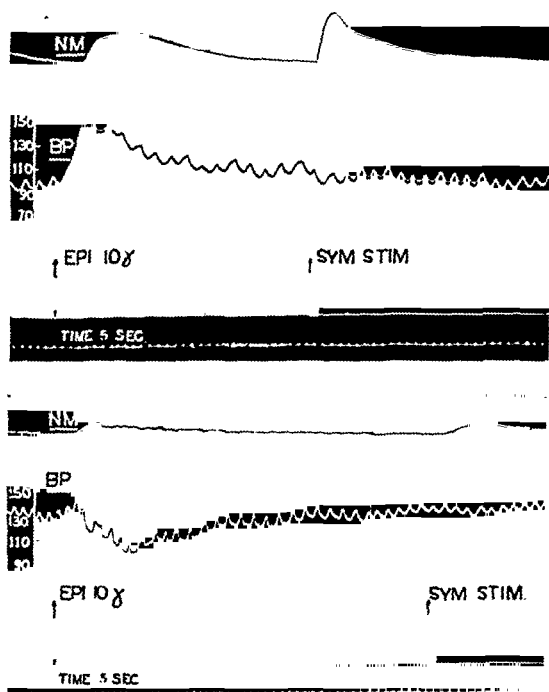


FIG. 1. Effect of N-ethyl-N-(2-chloroethyl) benzhydrylamine-HCl (SY-2) on the blood pressure and nictitating membrane responses to epinephrine and sympathetic nerve stimulation in the pentobarbitalized cat.

Upper record: Control response of carotid blood pressure and nictitating membrane following injection of epinephrine and faradization of the cephalic end of a cervical sympathetic nerve.

Lower record: Responses obtained 30 to 40 minutes after intravenous injection of SY-2 in a dose of 20 mgm./kgm. Note epinephrine reversal and almost complete blocking of the nictitating membrane response.

intravenous injections of epinephrine-HCl (10 or 20 microgm.), nitrogen re-breathing over soda lime to produce anoxic anoxia for one minute, and faradization of the peripheral end of a sectioned splanchnic nerve. Comparison of mean pressor responses recorded before drug treatment with the mean pressor or depressor responses obtained afterwards constituted the basis for determining the degree of diminution, blocking or reversal of responses. SY-2 was injected slowly intravenously (two min.) in a dose of 20 mgm./kgm. in the hopes that this dose

would be sufficient to provide complete adrenergic blockade. In view of the possibility that adrenal inactivation by itself would decrease the magnitude of the blood pressure responses, further control data were obtained by repeating the identical procedures in three dogs with adrenal veins ligated but not treated with SY-2.

In the three treated dogs, epinephrine reversal occurred consistently when injections were made from 10 to 120 minutes following injection of SY-2. During the same period nitrogen inhalation induced depressor responses; for example, the control mean pressor response of 16 mm. Hg was changed to a mean depressor response of -12 mm. Hg within one hour. Thus, epinephrine and anoxia both induced depressor responses after injection of SY-2. No such reversals occurred in the three control experiments in which no drug treatment was instituted, although pressor responses to anoxia and epinephrine were diminished by 30 per cent in these adrenal-inactivated dogs. Depressor responses to anoxia in these

TABLE 3

Comparison of adrenergic blocking and antihistamine action of 2-haloethylamines

TYPE OF COMPOUND	PHARMACOLOGICAL ACTION*	
	Histamine Antagonism	Adrenergic Blocking
1. N-Ethyl-N-(2-chloroethyl)-benzhydramine-HCl (SY-2)†	- or ±	+
2. N-Ethyl-N-(2-chloroethyl)-9-fluorenamine-HCl (SY-21)	-	++++
3. N-(2-Bromoethyl)-N-ethyl-1-naphthalene-methylamine-HBr (SY-28)	++++	++++
4. 2-(2-Biphenyloxy)-2'-chloro-triethylamine-HCl (SY-8)	++	++

* None of these compounds exert appreciable anti-acetylcholine action.

† Properties and potency nearly identical with those of Dibenzamine.

experiments with adrenal-inactivated dogs treated with SY-2 could scarcely be due to liberation of epinephrine. Depressor effects were undoubtedly due to direct effects of anoxia, cardio-vascular reflexes, and mechanical factors, that is, influences which usually tend to reduce blood pressure during anoxia but which are opposed by effects of adrenergic nervous activity including release of epinephrine.

Electrical stimulation of the peripheral end of sectioned splanchnic nerves induced a mean pressor response of 26 mm. Hg before drug treatment but only 7-10 mm. Hg from 10 to 120 minutes after treatment. That is, pressor responses resulting from direct stimulation of postganglionic adrenergic nerve fibers were not completely blocked. The diminution in response was not due to decreased sensitivity referable to adrenal inactivation because in the three untreated control dogs with adrenals inactivated nerve stimulation elicited undiminished mean pressor responses throughout the duration of the experiments (two to three hours). Nearly full blockade of pressor responses to splanchnic stimulation reveals that

SY-2 exerts a blockade which is probably at the effector cells and not at autonomic ganglia. Other evidence supporting this view is the reversal of pressor effects of injected epinephrine in cats and dogs as well as diminution and blockade of responses of the nictitating membrane in cats.

DISCUSSION. These studies indicate that of the alkyl homologues in a series of N-alkyl-N-(2-chloroethyl) benzhydrylamines the ethyl and isopropyl homologues were the most active adrenergic blocking agents. The ethyl homologue (SY-2) is not a potent adrenergic blocking drug, being no more potent than Dibenamine. Neither SY-2 nor Dibenamine exert appreciable anti-acetylcholine or anti-histamine activity, and from the information now available it appears that they are comparatively specific adrenergic blocking drugs.

A comparison of the properties of SY-2 with ethyl homologues in other series of 2-haloalkylethylamines which we have studied (4-11) will serve to emphasize differences in potency and specificity. Table 3 serves to characterize the ethyl homologues in the several chemical series. Although fairly specific, SY-2 has low potency when compared with N-ethyl-N-(2-chloroethyl)-9-fluorenamine (SY-21), the latter being a specific adrenergic blocking drug, and potent since doses of 1.0 mgm./kgm. and less will produce adrenergic blockade (11, 12). This fluorene compound differs from N-(2-bromoethyl)-1-naphthalenemethylamine (SY-28) which is a potent adrenergic blocking drug, but which is non-specific because it is a highly potent antagonist of histamine (4-6, 8). Thus, it is difficult to accurately classify SY-28 since it is a strong antagonist of both histamine and epinephrine. It is truly remarkable to note that SY-28 antagonizes these two substances which are themselves physiologically antagonistic with each other in a number of situations. Our studies also revealed that 2-(2-biphenyloxy)-2'-chlorotriethylamine (SY-8) was qualitatively similar to SY-28 but somewhat less potent with respect to antagonism of both histamine and epinephrine (9).

SUMMARY

In a series of N-alkyl-N-(2-chloroethyl)benzhydrylamines the ethyl and isopropyl homologues exerted a moderate degree of adrenergic blocking action. Higher alkyl homologues exhibited less activity. The ethyl homologue (SY-2) was found to be similar to Dibenamine with respect to potency, duration of action and specificity. Compounds in the series were found to be practically devoid of antihistamine and anti-acetylcholine properties.

SY-2 exhibited low oral acute toxicity in mice, comparatively small doses reduced the toxicity of epinephrine in mice and moderate doses failed to diminish histamine-induced bronchospasm in guinea pigs. In intravenous doses of 5.0 to 20.0 mgm./kgm., SY-2 induced epinephrine reversal, prevented pressor responses to anoxic anoxia and definitely reduced pressor responses to splanchnic stimulation in dogs. In cats, doses of 20.0 mgm./kgm., intravenously, induced epinephrine reversal and diminished or blocked responses of the nictitating membrane to injections of epinephrine and stimulation of the cervical sympathetic nerves. Properties of SY-2 have been compared or contrasted with those demonstrated for ethyl homologues in several other series of 2-haloalkylethylamines.

REFERENCES

1. LOEW, E. R., MICETICH, A., AND ACHENBACH, P.: Fed. Proc., **6**: 351, 1947.
2. NICKERSON, M., AND GOODMAN, L. S.: THIS JOURNAL, **89**: 167, 1947.
3. LOEW, E. R., KAISER, M. E., AND MOORE, V.: THIS JOURNAL, **83**: 120, 1945.
4. ACHENBACH, P., AND LOEW, E. R.: Fed. Proc., **6**: 304, 1947.
5. LOEW, E. R.: Physiol. Rev., **27**: 542, 1947.
6. LOEW, E. R.: Ann. N. Y. Acad. Sci., (in press), 1948.
7. LOEW, E. R., AND MICETICH, A.: THIS JOURNAL, **93**: 431, 1948.
8. LOEW, E. R., AND MICETICH, A.: THIS JOURNAL, **94**: 339, 1948.
9. LOEW, E. R., AND MICETICH, A.: THIS JOURNAL, **95**: 448, 1949.
10. STONE, C. A., AND LOEW, E. R.: THIS JOURNAL, **94**: 350, 1948.
11. LOEW, E. R.: Unpublished data.
12. KERWIN, J., ULLYOT, G. E., FELLOWS, E. J., AND MACKO, E.: Fed. Proc., **8**: 308, 1949.

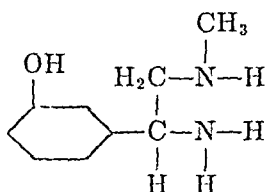
COMPARISON OF VASOMOTOR ACTIVITY OF 1-(m-HYDROXY-PHENYL)-N²-METHYLETHYLENE DIAMINE DIHYDROCHLORIDE (Nu-1683) WITH THAT OF EPINEPHRINE AND EPHEDRINE USING THE RAT MESO-APPENDIX TEST ^{1,2}

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Duschinsky, Dolan, Randall and Lehmann have recently developed a substance, Nu-1683³, 1-(m-hydroxyphenyl)-N²-methylethylene diamine dihydrochloride, which appears to have sympathomimetic properties (2, 3). Its structural formula is:



Nu-1683

This paper is a comparison of the vasomotor properties of this substance with those of epinephrine and ephedrine by means of the rat meso-appendix technique described by Chambers and Zweifach (4, 5).

METHODS. *Rat meso-appendix technique.* The meso-appendix of a rat, anesthetized with ethyl urethane (150 mgm./100 gm.) and weighing 90-150 gm., was exposed by a midline abdominal incision. The rat was placed on its right side on the rat board and the exteriorized meso-appendix was draped over a glass horseshoe and constantly bathed with a drip of 1 per cent gelatin in Ringer's solution, the temperature of which was adjusted to maintain the meso-appendix at 37-38°C. A precapillary or terminal arteriole with steady blood flow was observed and selected as the test vessel. The drugs were tested by intravenous injection and by topical application.

a. Intravenous injection. The effects of intravenous injections of ephedrine and Nu-1683 were compared as follows. Epinephrine was applied topically by dropping directly on the exteriorized mesentery three or four drops of a 1:2 million to 1:6 million dilution. It was determined at which dilution within this range a minimal constrictor response was obtained in the test vessel. The response by the vessel to the topical application of epinephrine was evaluated in terms of 1+ to 4+. Slight transient slowing of the flow through the vessel for 15-25 seconds following the application of epinephrine was designated as a 2+ response. The dilution which caused the 2+ response was checked two or three times, at three minute

¹ This work was supported by a grant from The Roche Anniversary Foundation, 475 Bloomfield Avenue, Montclair, N. J.

² A preliminary report of this work was presented at the 1948 meeting of the Society for Pharmacology and Experimental Therapeutics (1).

³ This drug was supplied by Dr. Elmer L. Sevringhaus of Hoffman-LaRoche, Inc., Nutley, N. J.

intervals, to insure an accurate calibration. Ephedrine or Nu-1683 dissolved in 0.5 cc. or less of saline, was then injected intravenously and topical application of epinephrine was continued at three minute intervals. This testing was continued until the reactivity of the vessel had returned to the calibration level, after which time the rat was discarded, since only one test was performed on a rat.

After the intravenous injection of either ephedrine or Nu-1683, it was found that a lower concentration of the topically applied epinephrine was required to produce the same 2+ vasoconstrictor response, i.e., the intravenously injected drug had an epinephrine augmentor effect. The magnitude of the augmentor effect was expressed as the ratio of the control dilution of epinephrine which produced the desired calibration response (2+) to the experimental dilution of epinephrine necessary to produce the same 2+ response by the vessel after the intravenous injection of the experimental drug. That is, if after the intravenous injection of ephedrine, a 1:6,000,000 concentration of topically applied epinephrine produced the same augmentor effect as the previous 1:4,000,000 concentration, a 1.5 fold effect ($1/4,000,000 \div 1/6,000,000 = 1.5$) was recorded for the ephedrine injection.

b. *Topical application.* In another series of experiments the responses of the blood vessels of the meso-appendix to direct topical application of Nu-1683 were observed and compared with those to the topical application of epinephrine and ephedrine. Using the set-up described above, application of three drops of one of these drugs was made through an 18-gauge needle with the drip turned off. The drip was resumed after a 30-second interval to prevent drying and cooling of the tissue. Observations were made continuously until flow through the vessel returned to normal. With this technique repeated tests were made of different concentrations of the three substances on the same animal. However, at least five minutes were allowed after full recovery from one test, before a second application was made. The responses were graded 1+ to 4+. One plus was used to indicate a just detectable narrowing of the muscular vessels (arterioles, precapillary sphincters and venules); 2+ signified definite narrowing of the vessel's with slowing of flow through them; 3+ meant marked narrowing of the vessel's with intermittent flow, particularly in the venous channels and 4+ indicated maximum constriction, with complete shutdown of flow.

It is felt by Zweifach (7) that consistent results can be obtained with the rat meso-appendix method only when care is taken to assure constant experimental conditions. These include keeping the rats, before and during the test, at an environmental temperature of 75 to 79°F., making up fresh solutions daily and the high dilution of epinephrine every few minutes and the avoidance of pyrogenic material in solutions and apparatus. During very warm weather we have occasionally been unable to obtain consistent results with the above technique. This was probably due principally to the lack of suitable air conditioning equipment since consistently satisfactory responses have occurred during the remainder of the year.

RESULTS. 1. *Intravenous injection. a. Ephedrine.* The responses in all experiments are summarized in table 1. Injection of 0.01 mgm./100 gm. of ephedrine caused a maximum augmentor effect of 1.5 to 2 and augmentation persisted, on the average, for 29 minutes. Ephedrine, 0.1 mgm./100 gm. caused on the average a 4.6 fold augmentor effect and the return to the control level occurred in approximately 42 minutes. The rise to the peak effect was rapid and maintained for a longer time than with the smaller quantity of ephedrine. One-half mgm./100 gm. caused complete shutdown of all vessels, which persisted longer than 60 minutes.

b. *Nu-1683.* All experiments are summarized in table 1. Injection of 0.01 mgm./100 gm. of Nu-1683 gave on the average a 2.35 fold augmentor effect, with return to normal in approximately 30 minutes. Injection of 0.1 mgm./100 gm. of Nu-1683 gave an augmentation of 2 to 4 fold with return to normal in 25 to more

than 60 minutes. After this dose the entire visible bed became sluggish and flow was slower. Following the topical application of epinephrine flow became still slower and the vessels appeared congested in the venous end before they did in the arteriolar end of the visible bed, and occasionally the flow of blood in the venules completely stopped for short intervals.

When 0.5 mgm./100 gm. and 1.0 mgm./100 gm. of Nu-1683 were administered the rise to the peak effect was rapid and steady. The vessel shut down with the last application of epinephrine (indicated by the sign ∞), and remained shut down for the remainder of the 60-minute period, after which time the rat was removed from the rat board. These animals did not show the waking effect which was occasionally noted in animals given similar amounts of ephedrine.

2. *Topical application to the meso-appendix.* The responses to the topical application of epinephrine, ephedrine and Nu-1683 to the rat meso-appendix are sum-

TABLE 1
Summary of Effects of Intravenous Injections

NO. OF RATS	DOSE/100 GM. BODY WEIGHT	EPINEPHRINE AUGMENTOR EFFECT		DURATION OF EFFECT MINUTES	
		Range	Average	Range	Average
Ephedrine					
10	mgm. 0.01	1.5 - 2	1.8	22 - 51	29.4
10	0.1	2 - 6	4.6	31 - >60	42
1	0.5	∞	∞	>60	>60
Nu-1683					
10	0.01	1.5 - 4	2.35	25 - 40	30.7
10	0.1	2 - 4	3	25 - >60	37
2	0.5	∞	∞	>60	>60
3	1.0	∞	∞	>60	>60

marized in table 2. The 1:6,000,000 and 1:4,000,000 dilutions of epinephrine caused 1+ to 3+ responses within approximately 17 seconds, the effects being detectable for around 25 seconds. The responses to higher concentrations showed similar constancy and demonstrate that the higher the concentration of epinephrine, the greater and longer-lasting is the response. Ephedrine and Nu-1683 applied topically had similar effects but required much higher concentration and the maximum effect appeared more slowly than with epinephrine.

DISCUSSION. The results of the intravenous injections demonstrate that these drugs have a vaso-exciter or epinephrine augmentor effect in that they enhance the reactivity of the small vessels to topical application of epinephrine. In this regard ephedrine and Nu-1683 are about equally effective. However, it is not clear, from the intravenous injections alone, whether the drugs act directly upon the blood vessels, or indirectly through effects upon the animals' blood pressure.

The results of direct topical application, however, confirm that Nu-1683 and ephedrine do directly affect the small blood vessels. Based upon this method of testing the order of dosage necessary to produce comparable effects is: epinephrine 1:1,000,000, Nu-1683 1:750 and ephedrine 1:500. All three act upon the arterioles, capillaries and venules.

During the course of these studies the effects of intra-arterial injections of these drugs upon the blood flow through the isolated blood perfused extremity were studied by Wayne *et al.* (6). The latter studies indicate also that these substances have a direct vasoconstrictor effect and that the order of potency is the same as that noted above.

TABLE 2
Summary of effects—topical applications

NUMBER OF APPLICATIONS	CONCENTRATION	EFFECT ON ARTERIOLES		TIME TO REACH MAXIMUM EFFECT SECONDS		TIME TO RETURN TO CONTROL STATE - MINUTES	
		Range	Av.	Range	Av.	Range	Av.
Epinephrine							
5	1:100,000	4+	4+	15-60	35	6-50	50
1	1:1,000,000	4+	4+	25	25	1 2	1 2
1	1:2,000,000	4+	4+	20	20	0.75	0.75
7	1:4,000,000	2+-3+	2.3+	12-20	17.5	0.25-0.55	0.4
7	1:6,000,000	1+-3+	2.1+	10-20	16.5	0.3-0.67	0.45
Ephedrine							
3	1:250	4+	4+	45-60	52	35-40	38
5	1:500	4+	4+	50-60	54	1.25-1.7	1.4
Nu-1683							
5	1:500	4+	4+	60-80	69	30->55	>37
6	1:750	4+	4+	55-75	64	1.25-3	1.8
4	1:1,000	3+-4+	3.5+	30-55	45	0.95-1.3	1.16

SUMMARY

The effects of intravenous injections and of topical applications of epinephrine, ephedrine and Nu-1683, a new sympathomimetic diamine, have been compared by noting their effects upon the vessels of the rat meso-appendix observed microscopically.

Intravenous injections of ephedrine and Nu-1683 augment approximately equally the vasoconstrictor effects of topically applied epinephrine.

Topical applications of all three substances cause local vasoconstriction involving arterioles, venules and capillaries which increases in intensity and duration with dose. Epinephrine, 1:1,000,000, Nu-1683, 1:750, and ephedrine, 1:500 produce comparable effects.

REFERENCES

1. KUNZE, D. C., BOBB, J. R. R., AND GREEN, H. D.: Fed. Proc., **7**: 235, 1948.
2. DUSCHINSKY, R., DOLAN, L. A., RANDALL, L. O., AND LEHMANN, G.: J. Am. Chem. Soc., **69**: 3150, 1947.
3. LEHMANN, G., AND RANDALL, L. O.: THIS JOURNAL, **93**: 114, 1948.
4. CHAMBERS, R., AND ZWEIFACH, B. W.: Am. J. Anat., **75**: 173, 1944.
5. ZWEIFACH, B. W.: Methods in Medical Research, V. R. Potter, Editor, The Year Book Publishers, Chicago, Ill., 1948, p. 131.
6. WAYNE, H., FRANKLIN, L., AND GREEN, H. D.: Fed. Proc., **8**: 345, 1949.
7. ZWEIFACH, B. W.: Personal communication.

SOME EFFECTS OF DIBENZYL- β -CHLORETHYLAMINE (DIBEN-AMINE) ON THE MAMMALIAN HEART

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It has been reported that dibenzyl- β -chlorethylamine (Dibenamine)³ does not interfere with the cardio-accelerator effect of epinephrine, but does prevent the ventricular irregularities produced by epinephrine in the presence of cyclopropane or other hydrocarbons (1). Little attention has been paid, however, to the effect of this adrenergic-blocking agent on the other actions of epinephrine on the heart. The present study was intended to broaden this part of our knowledge of the actions of dibenzyl- β -chlorethylamine by testing the effect of this compound on several preparations of cardiac tissue. The results fall into two categories, namely, (a) the absence of influence of dibenzyl- β -chlorethylamine on a number of effects of epinephrine on the heart, and (b) the effects of dibenzyl- β -chlorethylamine itself on the heart. In relation to the latter results, the adrenergic-blocking agent was compared with its close chemical relative, dibenzyl-ethanolamine, and with quinidine. A preliminary note has already been published (2). The compounds used were dissolved in saline acidified by the addition of hydrochloric acid. Dibenzyl- β -chlorethylamine hydrochloride was always freshly prepared.

1. *Dog heart-lung preparation.* Eight heart-lung preparations were performed according to the method of Patterson and Starling (3), using dogs weighing between 8 and 15 kgm., anesthetized by the intraperitoneal injection of sodium pentobarbital.⁴ Right and left auricular pressures, systemic output, pulmonary arterial pressure, and coronary flow were measured by methods described by Krayer and Mendez (4).

In three out of four experiments in which dibenzyl- β -chlorethylamine hydrochloride was administered in doses up to 45 mgm. (making potential concentrations in the heart, lungs, and blood up to 1:20,000), the performance of the heart remained normal during periods of observation up to 144 min. In the fourth experiment, 11 mgm. produced evidence of slight failure (rise of right auricular pressure) within one min. after the compound had been added to the blood in the venous reservoir. When 50 or more mgm. were administered, failure occurred uniformly in four experiments, as indicated by rise of right and left auricular pressures, fall of systemic output, and cardiac dilatation (fig. 1). The

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³ Kindly supplied by Dr. W. Gump, of Givaudan-Delawanna, Inc.: now available from Smith, Kline, and French, Philadelphia, Pa.

⁴ Nembutal Sodium, kindly supplied by Abbott Laboratories, North Chicago, Ill.

failure began shortly after the administration of the compound and became more severe during the following five to eight min.; during periods of observation up to 45 min., failure did not spontaneously disappear. When the failure was mild, the earliest change noted was a rise of pulmonary arterial pressure. When the total dose exceeded 150 mgm. (making a potential concentration of about 1:6000), the failure was severe.

Less than 138 mgm. of dibenzyl- β -chlorethylamine did not affect the rate of beating of the sinus node when observed over periods up to 110 min. In three

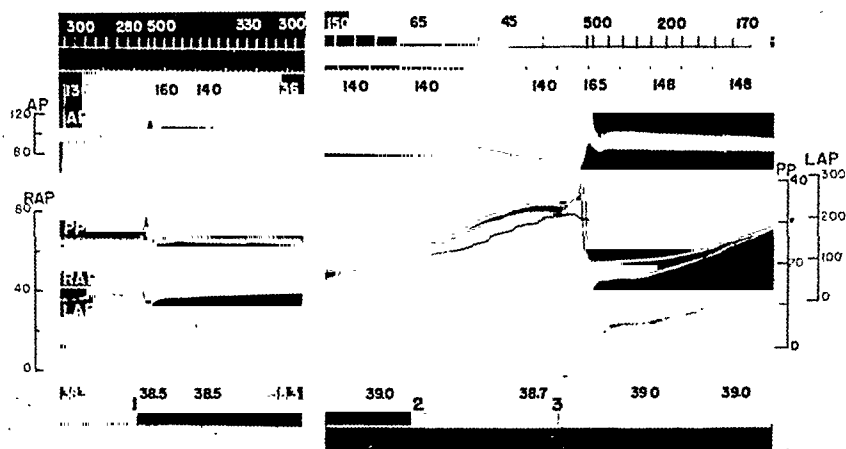


FIG. 1. EFFECTS OF EPINEPHRINE AND DIBENZYL- β -CHLORETHYLAMINE IN THE HEART-LUNG PREPARATION

Male dog, 10.3 kgm. Anesthesia, sodium pentobarbital, 35 mgm. per kgm. Arterial resistance, 80 mm. Hg. Weight of ventricles, 91 gm.

Tracings from top to bottom: Systemic output, each signal indicating 100 cc.; time in 10 sec. intervals; arterial pressure (scale on left in mm. of Hg); pulmonary arterial pressure (scale on right in cm. of water); right auricular pressure (scale on left in mm. of water); and left auricular pressure (scale on right in mm. of water).

Horizontal rows of figures from top to bottom: Systemic output in cc. per min.; heart rate per min.; blood temperature in degrees C.

Previous administration of 20 mgm. of sodium pentobarbital had induced mild failure. At signals 1 and 3, 2 microgm. of epinephrine were injected. Between the two segments of record, and 27 min. before the second segment, 50 mgm. of dibenzyl- β -chlorethylamine hydrochloride were added. At signal 2, this dose was repeated.

experiments, 138 to 250 mgm. produced within five to ten min. a 10 to 15 per cent slowing of sinus rate. In doses not producing failure the compound did not affect coronary sinus outflow.

The cardio-accelerator and positive inotropic effects of 1 to 10 microgm. doses of epinephrine were not decreased in intensity or duration by the previous administration of dibenzyl- β -chlorethylamine even when the latter produced failure and slowing of sinus rate. The coronary vasodilatation produced by epinephrine was evident after the blocking compound had been given (three experiments). The brief rise of pulmonary arterial pressure which follows the administration

of 1 to 10 microgm. of epinephrine was changed to a fall of similar duration and extent (three experiments).

II. *Isolated rabbit auricle.* The rabbit was killed by a blow on the neck; its chest was rapidly opened, and the auricle was removed and washed in Tyrode's solution. The auricle was then suspended in 50 cc. of Tyrode's solution at 25°C. continuously oxygenated by a copious stream of fine bubbles of air from a sintered glass filter. In the bath, the auricle lay in contact with four chlorided silver electrodes, two of which were used for the delivery of electrical stimuli (1 millisecc. square waves of twice threshold voltage from a Grass stimulator, model 3). The other two electrodes were used to lead the electrograms of the auricle to a Grass inkwriting oscillograph. Mechanograms were also recorded via a light lever on a smoked drum. At intervals during the experiment, the maximal rate of stimulation which the auricle would follow for ten seconds was determined by a procedure resembling that described by Dawes (5). The electrogram of the auricle proved to be a more reliable indicator of auricular response than the mechanogram. Solutions of the substances to be studied were added to the bath in volumes not exceeding 0.5 cc.

When dibenzyl- β -chlorethylamine hydrochloride solution was added to make a final concentration of 1:100,000 in the Tyrode's solution, the latter became cloudy, but gradually the solution again became clear. It can therefore be assumed that the concentration of the dissolved dibenzyl- β -chlorethylamine was less than the stated level, and that either binding or chemical change of this compound proceeded at a significant rate. The earliest change noted after adding the dibenzyl- β -chlorethylamine was a decrease in the maximal rate, when this was tested about fifteen min. later (fig. 2). The maximal rate fell without apparent latency and steadily over the period of one or two hours during which observations were made, from about 6 per sec. to 3 or 4 per sec. After a latency of 18 to 50 min., the rate at which the auricle beat when not stimulated began to fall; it then slowly and steadily decreased. Repeated washing did not alter either of these effects of dibenzyl- β -chlorethylamine.

When epinephrine was added to the bath to make a final concentration of 1 to 1 million, one to three hours after the dibenzyl- β -chlorethylamine had been added, it produced the same degree of effects on the auricle as it had before the addition of the antagonist, i.e., (a.) accelerated rate of the unstimulated auricle, (b.) increased amplitude of the mechanogram, and (c.) increased maximal rate.

III. *Auricular flutter in the dog.* In sixteen dogs, of 8 to 20 kgm. body weight, anesthetized by intraperitoneal injection of 0.7 cc. per kgm. of Dial-urethane solution⁶, auricular flutter was studied by the method of Rosenblueth and García Ramos (6). The heart was decentralized by vagotomy and removal of the stellates and the upper four pairs of thoracic ganglia. The animal was rotated on a longitudinal axis so that the heart fell to the left, making easier access to the right auricle. Small steel clips were applied to the auricle to stimulate and to record the auricular electrogram. The latter and the electrocardiogram were recorded simultaneously on two channels of a Grass inkwriting oscillograph. The auricle was stimulated at rates near the maximal rate which it will follow (10 to 15 per sec.), in the attempt to initiate auricular flutter. In one animal, stable flutter was established in this way. In the other animals, the auricle was crushed with hemostats so as to make a killed

⁶ Kindly supplied by Ciba Pharmaceutical Products, Inc., Summit, N. J.

area stretching from the region of the inferior vena cava to that of the auricular appendage as a possible site for a circus movement. The auricle was again stimulated, except in one animal in which crushing itself initiated stable flutter; in all but two animals in which this was done, and in all animals here reported, stable flutter was established, sometimes only after additional crushing.

Mean carotid arterial pressure exceeded 80 mm. of mercury during the control periods at the beginning of the experiments, but after the administration of dibenzyl- β -chlorethylamine, dibenzylethanolamine, or quinidine, arterial pressure usually fell to the range of 50 to 70 mm. In the control periods (13 to 70 min.) the auricles fluttered at average rates which varied in different animals

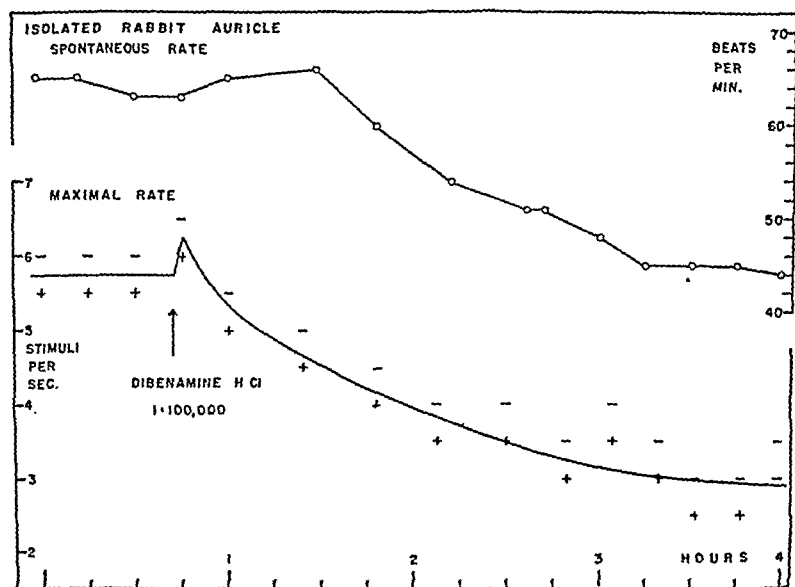


FIG. 2. EFFECTS OF DIBENZYL-B-CHLORETHYLAMINE ON THE ISOLATED AURICLE OF THE RABBIT

Upper curve: sinus rate. Lower curve: maximal rate (see text): plus marks indicate that the auricle followed the corresponding number of stimuli per sec.: minus marks indicate that it did not.

from 337 to 564 per min. Rates were determined by counting beats and estimating tenths of beats in 0.1 min. segments of the electrograms; average rates are based on counts at intervals averaging 2.2 min. (range 0.1 to 13 min.). In no animal did the rate of flutter vary in the control period by more than a standard deviation of 3.3 per cent. There was no obvious correlation among the variables, body weight, average rate of auricular flutter, standard deviation of this average, and duration of the control period. The ventricular rate was usually 1/2 of the auricular; this occurred at auricular rates of 324 to 515 per min. In two of eleven experiments, the ratio of ventricular to auricular beats was less than 1:2 for part of the time, at auricular rates of 502-522 and 359-393; and in seven

other experiments it was more than 1:2 for part or all of the time, at auricular rates up to 440 per min.

Dibenzyl- β -chloroethylamine; dibenzylethanolamine, and quinidine given intravenously in appropriate doses had similar effects on this preparation (fig. 3). As noted above, they usually lowered the arterial pressure. They caused a prompt decrease in the rate of beating of the fluttering auricle, with return to the original rate within 20 to 60 min. In association with this there was often a relative or absolute increase in the ventricular rate; but since at slower auricular rates the ventricle usually beats more rapidly in auricular flutter, no conclusions can be drawn about the effect of these agents on auriculo-ventricular conduction. During the fall of auricular rate shortly after the injection of the drug, the auricular flutter sometimes ceased and sinus rhythm prevailed; this

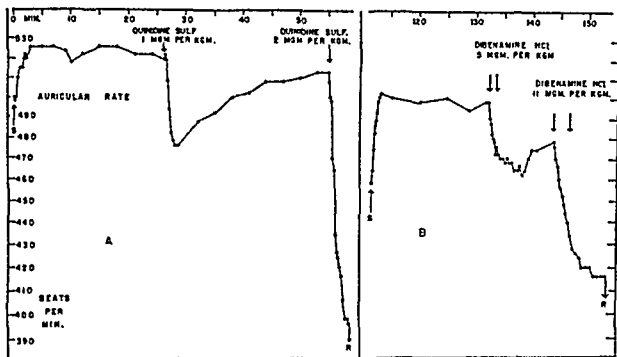


FIG. 3. EFFECT OF QUINIDINE AND DIBENZYL- β -CHLORETHYLAMINE ON THE RATE OF THE FLUTTERING AURICLE IN THE DOG

Abscissae: time in min. Ordinates: auricular beats per min. S indicates stimulation of the auricle, setting up the flutter. R indicates disappearance of the flutter and reversion to sinus rhythm.

occurred at auricular rates of 482 to 230. Usually flutter could be re-established by stimulation within a few minutes, when recovery from the effects of the drug was in progress.

In doses of 3 to 20 mgm. per kgm. dibenzyl- β -chloroethylamine hydrochloride diminished the rate of auricular flutter by 2 to 17 per cent, the effect increasing with dose in this range. In five experiments, quinidine sulfate had effects of similar extent and duration in doses twenty times smaller (fig. 3). In four experiments dibenzylethanolamine hydrochloride in doses of 10 to 20 mgm. per kgm. produced 26 to 45 per cent reductions in rate of flutter; these effects were of the same duration as the smaller effects produced by 10 to 20 mgm. per kgm. of dibenzyl- β -chloroethylamine hydrochloride.

Epinephrine in doses of 5 to 100 microgm. accelerated the auricular flutter

for two to five min. (four experiments), as illustrated in fig. 4. The auricular rate increased as much as 10 per cent in some instances. The ventricular rate, previously $1/2$ that of the auricles, accelerated far out of proportion to the increase of auricular rate (up to 50 per cent more than the previous ventricular rate), and sometimes for a longer period than that of the auricular acceleration. Since the ventricular complexes of the electrocardiogram remained supraventricular during this phenomenon, it is clear that the ventricular acceleration cannot be attributed to ventricular ectopic foci but must be attributed to an improve-

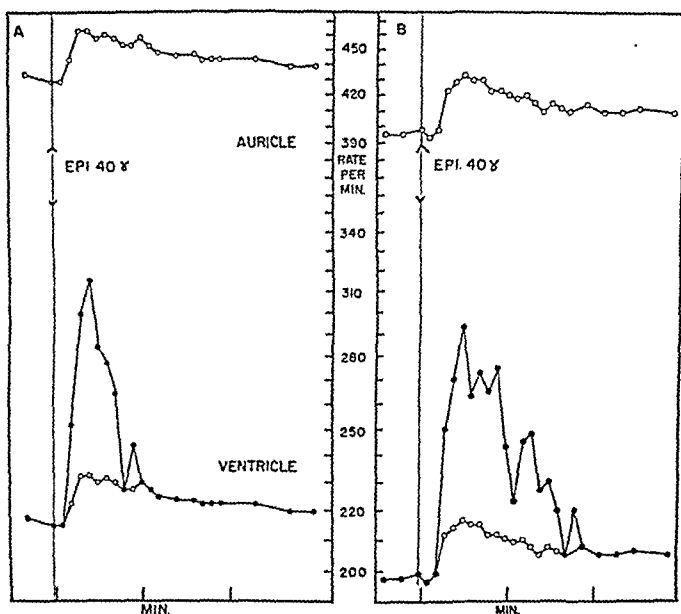


FIG. 4. EFFECT OF EPINEPHRINE ON AURICULAR AND VENTRICULAR RATES IN AURICULAR FLUTTER IN THE DOG; ABSENCE OF ACTION OF DIBENZYL- β -CHLORETHYLAMINE ON THESE EFFECTS

Upper curve: auricular rate. Lower curves: dots, actual ventricular rate; circles, half the auricular rate. The increase of ventricular rate over one-half the auricular rate represents improvement in a-v conduction. In this and the preceding figure, rates are represented on logarithmic scales. After A and 11 min. before B, 20 mgm. per kgm. of dibenzyl- β -chlorethylamine were administered.

ment of a-v conduction produced by epinephrine. The previous administration of dibenzyl- β -chlorethylamine or dibenzylethanolamine in doses of 10 to 20 mgm. per kgm. did not decrease the response of auricular or ventricular rates to subsequent doses of epinephrine given as long as 30 to 60 min. later. In the case of dibenzyl- β -chlorethylamine, the blood pressure response to epinephrine was reversed. The ventricular acceleration in response to epinephrine was sometimes larger after these amines (fig. 4 B), but this can be attributed to the slower rates of auricular flutter produced by the latter.

IV. *Cyclopropane-epinephrine ventricular irregularities.* Epinephrine was ad-

ministered to dogs under cyclopropane anesthesia in the customary manner (7). This procedure was carried out at various intervals after the intravenous administration of 15 or 20 mgm. per kgm. of dibenzyl- β -chlorethylamine hydrochloride in a series of six animals. In each animal the protection afforded against ventricular irregularities (detected electrocardiographically) eventually disappeared. In all instances, the protection was still great at the end of 24 hrs.; in two instances, it was evident after 48 hrs.; and in one of these two, it was still apparent after 67 hrs.

In six experiments in which the injection of epinephrine was performed under cyclopropane anesthesia as above, dibenzylethanolamine was injected in doses of 12 to 20 mgm. per kgm. twelve sec. to one hr. before the epinephrine injection. It exerted no apparent protection against the ventricular irregularities.

DISCUSSION. A. *Effects of dibenzyl- β -chlorethylamine on the heart.* In the preparations used dibenzyl- β -chlorethylamine depresses several aspects of cardiac function at concentrations ranging from 1:7000 to 1:330,000 (sections I, II, III). Hunt (8) found that 1:50,000 did not influence the contraction of the isolated papillary muscle. Considering the differences among these preparations, these concentrations are probably of little comparative significance. It is to be noted, however, that the dose which diminishes the rate of auricular flutter in the dog is relatively small when compared to doses having a striking action upon the pressor response to epinephrine (9). In the same preparation, doses of dibenzyl- β -chlorethylamine six times greater (20 mgm. per kgm.) did not have a detectable effect on auriculo-ventricular conduction. The doses producing heart failure (50 mgm. per kgm. or more) and decreasing sinus rate (138 mgm. per kgm. or more) in the heart-lung preparation, on the other hand, must be considered high.

The auricular and ventricular inotropic effects, the sinus rate unaffected by nervous influences, and a-v conduction were studied directly in the preparations used. The basic factors determining maximal rate as studied in the rabbit auricle are the rates of recovery of excitability and conduction.

Auricular flutter produced by the method of Rosenblueth and García Ramos has been demonstrated to be a circus movement (10). Since in the experiments reported here the size of the lacunae around which the circus may be rotating is not progressively changed, a progressive change of rate of flutter represents a proportionate change of auricular conduction velocity. The latter may, however, be conditioned by a lengthening or shortening of the recovery process, for at frequencies of 300 or more per min., each auricular wave is impinging upon the relatively refractory period of the preceding wave. Reversion from circus movement to sinus rhythm indicates that the wave met with refractory tissue and was therefore extinguished. This refractoriness could be due either to the interposition of ectopic beats or to sufficient lengthening of the refractory period of the auricular tissue without corresponding slowing of the conduction velocity. Since none of the substances studied which produces reversion from flutter to sinus rhythm initiated ectopic beats at the time of reversion, or at other times, the former possibility is excluded. No crucial evidence is available from these

experiments, however, that these substances did lengthen refractory period in the auricle. It is to be noted that reversion, though always associated with a decrease in the rate of flutter, occurred sometimes at relatively high rates and slight decreases in the rate of flutter and other times at relatively low rates and marked decreases. Hence there was little correlation between the decrease of conduction velocity presumed to account for the slower rate of flutter and the degree of lengthening of the refractory period necessary for extinction of the circus.

B. *Dibenzyl- β -chlorethylamine versus epinephrine.* Epinephrine influences all the above aspects of cardiac function—inotropic, chronotropic, bathmotropic, and dromotropic—in a positive sense; yet, whereas dibenzyl- β -chlorethylamine is capable of interfering with the contractile effect of epinephrine upon smooth muscle, it did not affect several positive actions of epinephrine upon cardiac muscle. It has previously been reported that dibenzyl- β -chlorethylamine does not prevent the cardio-accelerator effect of epinephrine. The literature dealing with the other adrenergic-blocking compounds contains similar statements (1). It is clear that in most instances (but cf. 11) doses of adrenergic-blocking compounds large enough to abolish or reverse the pressor response to epinephrine do not affect its accelerator and inotropic actions. For dibenzyl- β -chlorethylamine the results reported above establish that this is true also for the effects of epinephrine on the maximal rate of the isolated rabbit auricle, and on auriculo-ventricular conduction and rate of auricular flutter in the dog. The absence of an anti-adrenergic effect might, however, be considered due merely to insufficient quantity of blocking agent or insufficient time of exposure to it. Indeed, for each adrenergic-blocking compound which was well-known before the advent of dibenzyl- β -chlorethylamine, competent observers have reported that sufficiently large doses do produce a diminution or abolition, or even a reversal, of the accelerator action of epinephrine in the mammalian heart (11-17). In the heart-lung preparation, at least, the doses of dibenzyl- β -chlorethylamine and the times of exposure are both large (from 20 min. after more than 200 mgm. per kgm. to 165 min. after more than 40 mgm. per kgm.). The size of dose one may use is limited by the deleterious effects of large doses on the heart. For present purposes it is enough to state that large doses of this compound do not interfere with the cardio-accelerator or positive inotropic effects of epinephrine.

C. *Comparison of dibenzyl- β -chlorethylamine with dibenzylethanolamine and quinidine.* Dibenzyl- β -chlorethylamine is effective against the excitatory actions of epinephrine upon smooth muscle by virtue of its β -chlorethylamine structure (1). Yet it may be expected to share some of the pharmacological properties of other amines lacking this structure but otherwise similar. Of particular interest is dibenzylethanolamine, which is thought to be one of the breakdown products of the β -chlorethyl compound. Dibenzylethanolamine is devoid of adrenergic-blocking activity (1), and unable to protect against cyclopropane-epinephrine ventricular irregularities, as noted above in confirmation of Nickerson and Nomaguchi (19). Yet its action on the rate of the fluttering auricle in the dog is similar to, though more intense than that of dibenzyl- β -chlorethylamine.

Other substances closely related but not known to have adrenergic-blocking properties are dibenzylethylamine and dibenzylmethylamine. Like dibenzyl- β -chlorethylamine, however, these have been found to raise the faradic fibrillation threshold of the rabbit's auricle and ventricle (20, 21), and the methyl compound has been reported to prevent chloroform-epinephrine ventricular fibrillation in the dog (22). These actions are not different from those produced by such more remotely related amines as procaine and quinidine, which affect numerous phases of the activity of different kinds of cardiac tissues.

A rational classification of the effects of substances like quinidine and dibenzyl- β -chlorethylamine on the heart is not yet available. One compares them and finds that they are alike in a number of ways. Quinidine is used as a point of reference not because any theoretical importance is attached to it but because relatively much is known about quinidine. The hypothesis which underlies the comparison is that quinidine and dibenzyl- β -chlorethylamine owe their similar actions to similar mechanisms. This remains to be established.

D. Onset and duration of effects of dibenzyl- β -chlorethylamine. Nickerson and Goodman (9) noted that the action of dibenzyl- β -chlorethylamine upon the pressor response to epinephrine reaches its maximum only after about 30 min. Since distribution through the body occupies but a few minutes, this time is probably taken up by the slow reaction of the blocking agent with the cellular elements. The results noted above in the rabbit auricle (fig. 1) might be interpreted in like manner, were it not for the fact that the addition of the dibenzyl- β -chlorethylamine hydrochloride solution to the bath led to a cloudiness of the latter, which slowly cleared. Presumably during this time not all of the dibenzyl- β -chlorethylamine was available to react with the auricular tissue. By contrast the effects of this compound on the ventricular muscle and the sinus node in the heart-lung preparation (fig. 1) and its effect on the rate of auricular flutter in the dog (fig. 3) reach a maximum in relatively few minutes.

When dibenzyl- β -chlorethylamine is injected intravenously in a cat, its effects on the pressor response to epinephrine can still be detected one and one-half to five days later (9). Inability to wash out the adrenergic-blocking effects of dibenzyl- β -chlorethylamine has been demonstrated in the isolated uterus of the rabbit (23, 8, 1). These facts suggest that the reactions of the agent with some tissues are not readily reversible. A similar lack of reversibility is reflected by the failure of repeated washing to restore to its normal state the rabbit auricle which has been exposed to this compound (section II). In the dog heart-lung preparation, failure produced by this agent did not show signs of spontaneous recovery for as long as 45 min. (section I); but, in view of Hunt's report that adrenergic-blocking material may remain in the blood of the cat for as long as five hours after the intravenous injection of 20 mgm. per kgm. of dibenzyl- β -chlorethylamine (8), one is not justified in judging the reversibility of the reaction producing the negative inotropic effect in the experiments here reported. In both of these instances, however, the reaction is not such as to cause adrenergic blockade, i.e., epinephrine produces its usual positive inotropic, chronotropic, and dromotropic effects. In the case of the rabbit auricle, therefore,

it is clear that dibenzyl- β -chlorethylamine can react in a difficultly reversible manner without producing an adrenergic-blocking effect. It is not surprising that such a reactive substance as the imino derivative of dibenzyl- β -chlorethylamine should enter into firm combination with more than one kind of tissue element. In contrast to the long duration of these effects is the brief action of injected dibenzyl- β -chlorethylamine on the rate of the fluttering auricle. The reaction of the compound with the tissue elements which leads to this effect is a readily reversible one.

The protection against cyclopropane-epinephrine ventricular irregularities has been reported to last at least 24 hrs. (19). The results noted in section IV indicate more fully that the duration of the protection is similar to that of the anti-pressor effect of dibenzyl- β -chlorethylamine, that is, one and one-half to five days. The same result obtains for the protection of unanesthetized dogs against the ventricular tachycardia produced by large doses of epinephrine in the absence of hydrocarbon sensitizer (24). It has been argued that the simultaneity of disappearance of these two effects, protection against irregularities and anti-pressor action, indicates that the former results from an adrenergic-blocking effect (1). This is supported by the fact that adrenergic blockade removes a factor which contributes to the production of ventricular irregularities, namely, the rise of arterial pressure usually produced by the epinephrine (25). A direct myocardial action of dibenzyl- β -chlorethylamine has, however, also been invoked to account for the protection (1). It is worth pointing out that (a) as yet no specific myocardial effect of dibenzyl- β -chlorethylamine has been demonstrated to produce this protection and (b) prolonged action is not necessarily adrenergic-blocking action, since the isolated rabbit auricle shows a difficultly reversible effect of dibenzyl- β -chlorethylamine which is not related to adrenergic blockade.

SUMMARY

1. Dibenzyl- β -chlorethylamine in high doses has a negative inotropic effect upon the heart-lung preparation of the dog. In still higher doses it decreases sinus rate.

2. It progressively diminishes the sinus rate and the maximal rate of the isolated rabbit auricle.

3. In doses of 3 to 20 mgm. per kgm. it decreases the rate of the fluttering auricle in the dog. Quinidine has a similar effect in doses twenty times smaller. Dibenzylethanolamine in equal doses has a more intense effect of similar duration.

4. The effects of epinephrine upon these preparations (positive inotropic effect, increase of sinus rate, increase of maximal rate, increase of rate of fluttering, improvement of AV conduction) are not affected by dibenzyl- β -chlorethylamine.

5. The protection against cyclopropane-epinephrine ventricular irregularities produced by dibenzyl- β -chlorethylamine lasts one and one-half to five days. Dibenzylethanolamine does not produce this protection.

REFERENCES

1. NICKERSON, M.: *Pharmacological Reviews*, **1**: 27, 1949.
2. ACHESON, G. H., FARAH, A., AND FRENCH, G. N.: *Fed. Proc.*, **6**: 305, 1947.
3. PATTERSON, S. W., AND STARLING, E. H.: *J. Physiol.*, **48**: 357, 1913.
4. KRAYER, O., AND MENDEZ, R.: *THIS JOURNAL*, **74**: 350, 1942.
5. DAVIES, G. S.: *Brit. J. Pharmacol.*, **1**: 90, 1946.
6. ROSENBLUTH, A., AND GARCÍA RAMOS, J.: *Arch. Inst. cardiol. México*, **17**: 1, 1947; *Am. Heart J.*, **33**: 677, 1947.
7. NICKERSON, M., SMITH, S. M., AND GOODMAN, L.: *Fed. Proc.*, **5**: 195, 1946.
8. HUNT, C. C.: *THIS JOURNAL*, **95**: 177, 1949.
9. NICKERSON, M., AND GOODMAN, L.: *THIS JOURNAL*, **89**: 167, 1947.
10. ROSENBLUTH, A., AND GARCÍA RAMOS, J.: *Arch. Inst. cardiol. México*, **17**: 441, 1947.
11. BOVET, D., AND SIMON, A.: *Compt. rend. Soc. de Biol.*, **119**: 1333, 1935.
12. DALE, H. H.: *J. Physiol.*, **34**: 163, 1906.
13. DEVLEESCHOUWER, G.: *Arch. internat. de pharmacodyn. et de thérap.*, **50**: 251, 1935.
14. BOVET, D., FOURNEAU, E., TRÉFOUEL, J., AND STRICKLER, H.: *Arch. internat. de pharmacodyn. et de thérap.*, **62**: 231, 1939.
15. JOURDAN, F., AND FROMENT, R.: *Compt. rend. Soc. de Biol.*, **130**: 1264, 1939.
16. MARTIN, J., AND LISSAK, K.: *Arch. f. exper. Path. u. Pharmacol.*, **196**: 1, 1940.
17. GUNN, J. A.: *J. Physiol.*, **100**: 64, 1941.
18. HAZARD, R., BEUVALLET, M., AND GIUDICELLI, R.: *Compt. rend. Soc. de Biol.*, **141**: 123, 1947.
19. NICKERSON, M., AND NOMAGUCHI, G. M.: *THIS JOURNAL*, **95**: 1, 1949.
20. MOISSET DE ESPANES, E., AND WEKSLER, B.: *Proc. Soc. Exper. Biol. & Med.*, **63**: 195, 1946.
21. DiPALMA, J., AND LAMBERT, J. J.: *Science*, **107**: 66, 1948.
22. CHARLIER, R., AND PHILIPPOT, E.: *Compt. rend. Soc. de Biol.*, **141**: 962, 1947.
23. ACHESON, G. H., AND FARAH, A.: Unpublished results.
24. WILBURNE, M., KATZ, L. N., ROBBARD, S., AND SURTSHIN, A.: *THIS JOURNAL*, **90**: 215, 1947.
25. MOE, G. K., MALTON, S. C., RENNICK, B. R., AND FREYBURGER, U. A.: *THIS JOURNAL*, **94**: 319, 1948.

MICROSCOPIC STUDIES ON VASCULAR REACTIONS TO ERGONOVINE MALEATE IN THE LIVING ANIMAL^{1,2}

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Ergonovine, an alkaloid base of ergot, was first isolated in 1935. It is used clinically in the treatment of postpartum hemorrhage, and also for the relief of migraine headache, which is generally conceded to be in some way related to vascular conditions.

A review covering the discovery, physical and chemical properties and therapeutic uses of ergonovine has been published by Smith (1). The unique clinical importance of this drug has been emphasized particularly by Dale (2), Dudley and Moir (3), Moir (4) and Adair, Davis, Kharasch and Legault (5).

Thus far there are no published reports on the vascular effects of ergonovine in which the arterioles, venules and the minuter vessels in the capillary bed have been studied directly. The present report deals with such direct observations, which were made upon newly formed blood vessels in transparent chambers in rabbits' ears. Such chambers have been described in numerous previous papers published from this department, under the supervision of Dr. Eliot R. Clark. The actual observations described in this paper were made with the use of the "moat chamber" (6, 7).

METHODS. For information concerning the construction, installation and use of the moat chamber, reference should be made to the original description by Abell and Clark (6). The response of the vessels in such chambers to ergonovine maleate was studied at magnifications of 200 and 400 diameters, and their reactions recorded photomicrographically with a Leitz 'mifilmca' camera. Measurements of the diameters of the vessels before and after the injections were made with a micrometer ocular.

Seven rabbits were used, each with a chamber in one ear. A total of 103 injections were made, and the effects of each of the concentrations used verified by repeating the experiments on different days, and in different rabbits. All of the injections were made into the marginal ear vein of the ear that did not contain the chamber.

The rabbits weighed between 3 and 4 kgm., and the doses are recorded in terms of total milligrams injected. Amounts varying from 0.005 mgm. to 4.0 mgm. were injected in 0.5 cc. to 1.0 cc. of mammalian Ringer's solution. Injections of mammalian Ringer's solution were made to see whether any caliber changes occurred as the result of the injection itself. No changes were observed.

OBSERVATIONS. I. Reactions of the Blood Vessels and Blood Flow to Injections of Ergonovine Maleate. 1. Arterioles. Injections of ergonovine maleate caused

¹ The ergonovine maleate used in these experiments was 'Ergotrate' (Ergonovine Maleate, U. S. P., Lilly).

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arteriolar constriction. The degree of constriction, the duration of constriction, and the effect upon the velocity of blood flow was proportional, within limits, to the amount injected, as shown in table 1. As is evident from this table, small amounts of ergonovine (0.005 mgm.) caused only slight arteriolar constriction, with little change in velocity of blood flow, and with return of the vessels to their control diameters within approximately five minutes. Larger injections (0.10-0.20 mgm.) produced greater constriction, cf. figs. 1 to 3. Following injections of still larger amounts (3.0 to 4.0 mgm.), the constriction persisted for a much longer time (three to five hours), and caused prolonged reduction in rate

TABLE 1

The effect of single injections of ergonovine maleate upon small arterioles and upon the velocity of blood flow

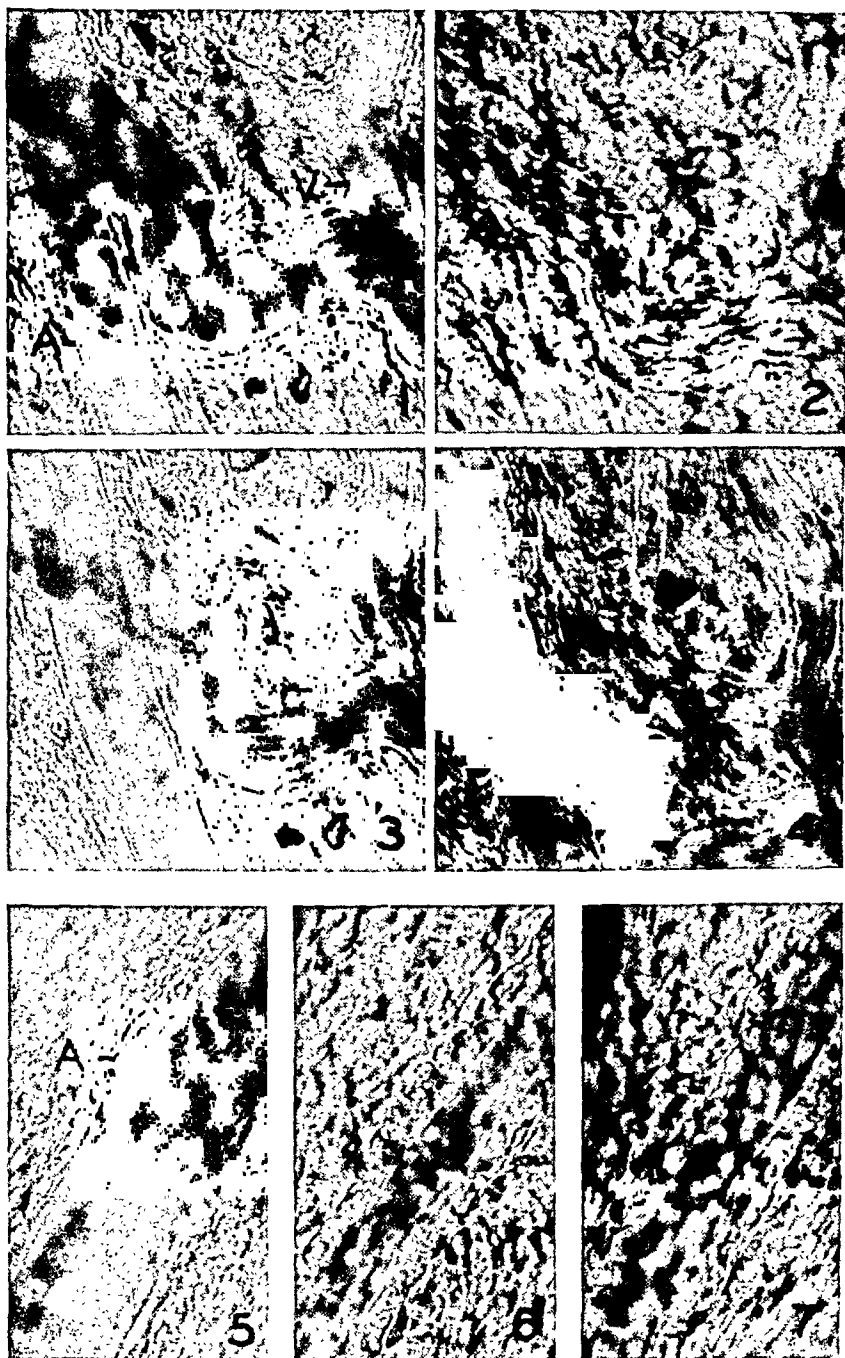
The figure "100" for degree of maximal constriction in per cent of control diameter means that the arteriole lumen was entirely obliterated. The figures given represent average results, the number of injections upon which the average is based for each amount of ergonovine injected being given in the left hand column.

NO. OF INJECTIONS	AMT. OF ERGONOVINE MALEATE INJECTED	CONTROL DIAMETER OF VESSEL	DURATION OF MAXIMAL CONSTRICTION	DEGREE OF MAX CONSTRICTION IN PER CENT OF CONTROL DIAM	EFFECT OF MAX. CONSTRICTION ON BLOOD FLOW	BEHAVIOR OF BLOOD FLOW DURING RETURN OF VESSELS TO THEIR CONTROL DIAMETERS	TIME OF RETURN OF VESSELS TO CONTROL DIAM., MIN AFTER INJECTION
	mgm.	microns	min.				
12	0.005	20	$\frac{1}{2}$	10-30	slight reduction	fairly rapid	3-5
10	0.01	20	$\frac{1}{2}$ -1	20-40	considerable reduction	very slightly reduced	5-7
14	0.10	20	$\frac{1}{2}$ -1 $\frac{1}{2}$	100	flow stopped	slightly reduced	10-12
33	0.20	20	1-1 $\frac{1}{2}$	100	flow stopped	moderately reduced	10-15
8	1.00	20	1 $\frac{1}{2}$ -2	100	flow stopped	markedly reduced	30-60
5	2.00	20	2-3	100	flow stopped	markedly reduced	90-120
10	3.00	20	3-4	100	flow stopped	sluggish	180-240
7	4.00	20	3 $\frac{1}{2}$ -5	100	flow stopped	sluggish	300

of blood flow. Figures 8 and 9 illustrate continued arteriolar constriction following injection of 3.0 mgm.

Injections of a given amount of ergonovine maleate made on successive days caused the same degree of constriction each day. Thus 0.01 mgm. (an amount in the rabbit equivalent by weight to the clinical dose of 0.2 mgm. in man) was injected on each of fourteen successive days, with similar results each day. At no time during or after this fourteen-day period was there any sign of injury to the vessels, and at the end of the fourteen-day period the vessels differed in no detectable way from their condition before the injections.

Repeated injections at intervals of fifteen to thirty minutes produced approximately the same degree of constriction each time when concentrations of 0.005 to 0.01 mgm. were used, but not with concentrations of 0.2 mgm. or more. As



Figs 1-7

shown in table 2, repeated injections of 0.2 mgm. at fifteen-minute intervals caused progressively less constriction of all arterioles up to 20 microns in diameter (cf. fig. 4). The fourth, fifth, and sixth injections produced actual dilatation of such arterioles. Although such smaller arterioles did not constrict after the second injection of 0.2 mgm., arterioles 45 microns in diameter and over did (cf. figs. 5 and 7, and table 2).

A single injection of 1.0 mgm. caused arterioles up to 20 microns in diameter to be unresponsive to a second injection made thirty minutes later, but arterioles 45 microns in diameter and over still constricted. The second injection made such large arterioles unresponsive to a third injection made thirty minutes after the second, but the main artery of the ear (1.0 mm. in diameter) constricted 90 per cent of its control diameter. The smaller arterioles actually dilated following this third injection, which caused simultaneous constriction of the main artery of the ear.

Thus the smaller arterioles became refractory to repeated injections of ergonovine maleate sooner than the larger ones; the number of injections required to produce refractoriness in a given arteriole depending upon the amount injected and the size of the arteriole.

Although repeated injections of 0.2 mgm. or more of ergonovine maleate rendered the smaller arterioles unresponsive to further injections of ergonovine, the response to epinephrine was not altered (cf. figs. 10-11). Thus following injections of 0.025 mgm. of epinephrine the arterioles constricted just as vigorously and stayed constricted just as long when they were refractory to ergonovine maleate as they did before ergonovine. This was found to be true for injections of ergonovine maleate up to 3.0 mgm. The effect of larger amounts upon the response of the arterioles to epinephrine was not tested.

2. *Venules.* Ergonovine maleate in amounts of 0.005 mgm. produced no appreciable effects upon the diameters of the venules. In amounts of 0.01 mgm.,

FIG. 1. Photomicrograph of arteriole (A) and venule (V) in part of the bay of a transparent "moat chamber" in a rabbit's ear, showing control appearance of vessels. Actual diameter of arteriole 20 microns. Mag. $\times 500$.

FIG. 2. Same vessels 45 seconds after intravenous injection of 0.2 mgm. of ergonovine maleate. Arteriole markedly constricted, and blood flow stopped. Venule partially constricted. Mag. $\times 500$

FIG. 3. Same vessels twelve minutes after injection. Arteriole somewhat dilated, due to reactive hyperemia. Venule still narrowed. Mag. $\times 500$.

FIG. 4. Same vessels 45 seconds after the last of three injections of 0.2 mgm. of ergonovine maleate, made at fifteen-minute intervals. Arteriole not constricted. Mag. $\times 500$

FIG. 5. Photomicrograph of a larger arteriole (A) in the bay of the same chamber, showing control diameter. Actual diameter of this arteriole 45 microns. Mag. $\times 500$.

FIG. 6. Photomicrograph of same arteriole one minute and fifteen seconds after first injection of 0.2 mgm. ergonovine maleate. Arteriole is markedly constricted, though not to as great a degree as the smaller arteriole shown in fig. 2, 45 seconds after this injection. Mag. $\times 500$.

FIG. 7. Photomicrograph of the same arteriole one minute and fifteen seconds after the third injection of 0.2 mgm. of ergonovine maleate, made at fifteen-minute intervals. This larger arteriole constricted markedly after the third injection, whereas the smaller arteriole shown in fig. 4 failed to constrict 45 seconds after this same injection. Mag. $\times 500$.

venules 20-35 microns in diameter constricted approximately 20 per cent, in amounts of 0.2 mgm. 30 per cent (cf. fig. 3), and in amounts of 4.0 mgm. also approximately 30 per cent.

3. *Capillaries.* No active capillary constriction was observed after any of the injections, the lumens of the capillaries remaining open at all times.

II. *Effect of Injections of Ergonovine Maleate upon the Blood Vascular Endothelium.* Ergonovine maleate, when injected into the rabbit in amounts comparable to those used clinically (i.e., in amounts of 0.01 mgm. in a rabbit, which is comparable to 0.2 mgm. in man), caused no irritating or injurious effect. There was no change in distribution of the blood platelets within the blood

TABLE 2

Effect of repeated injections of ergonovine maleate upon the diameters of small and medium sized arterioles, and upon the main artery of the ear

The injections were made at fifteen-minute intervals. The figure "100" for degree of maximal constriction in per cent of control diameter means that the arteriolar lumen was entirely obliterated.

AMOUNT OF ERGONOVINE MALEATE INJECTED	NO. OF INJECT.	SIZE OF VESSEL #1	DEGREE OF MAXIMAL CONSTRICTION IN PER CENT OF CONTROL DIAM.	SIZE OF VESSEL #2	DEGREE OF MAXIMAL CONSTRICTION IN PER CENT OF CONTROL DIAM.	SIZE OF VESSEL #3	DEGREE OF MAXIMAL CONSTRICTION IN PER CENT OF CONTROL DIAM.
<i>mgm.</i>		<i>microns</i>		<i>microns</i>		<i>microns</i>	
0.2	1	20	100	45	83		
	2	20	20	45	75		
	3	20	0	45	70		
	4	20	10% dilated				
	5	20	20% dilated				
	6	20	50% dilated				
1.0	1	20	100	45	100	1000	90
	2	20	0	45	40	1000	90
	3	20	20% dilated	45	0	1000	90

stream, no change in distribution or shape of the erythrocytes, no increase in stickiness of the endothelium toward leukocytes, no change in the leukocytes themselves. This was found to be true both of daily injections over a period of fourteen days, and also following repeated injections made on the same day. This was true even when the amount injected was 0.2 mgm. (twenty times greater than the comparative clinical dose in man).

Single injections of 3.0 mgm. (300 times the comparative clinical dose) were found not to increase appreciably the stickiness of the endothelium toward leukocytes (cf. figs. 8 and 9). Repeated injections of such extremely large amounts over a period of five days, however, caused marked sticking of leukocytes to the walls of the arterioles, capillaries and venules in two of the rabbits, the de-

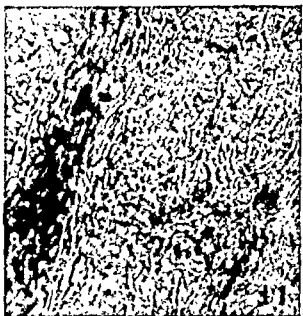


FIG. 8. Photomicrograph of arteriole (A) and vein (V) in a part of the bay of a "moat chamber" in a rabbit's ear, showing control appearance of vessels. Mag. $\times 500$.

FIG. 9. Same vessels one hour and sixteen minutes after intravenous injection of 3.0 mgm. of ergonovine maleate. Arteriole still constricted to approximately one-half its control diameter. No increase in adherence of leukocytes to the endothelium. Mag. $\times 500$.

FIG. 10. Photomicrograph of an arteriole and two venules in the bay of a "moat chamber", taken fourteen minutes following the last of four injections of 0.2 mgm. ergonovine maleate. The fourth injection produced no arterial constriction, and at the time this photograph was taken, the arteriole was completely refractory to ergonovine. Mag. $\times 500$.

FIG. 11. The same vessels 45 seconds following intravenous injection of 0.025 mgm. epinephrine. This injection was made fifteen minutes following the fourth injection of ergonovine, at which time the arteriole was refractory to ergonovine. The epinephrine has caused typical arteriolar constriction, with interruption of the blood flow. Mag. $\times 500$.

gree of sticking reaching phase 3 to 4 of the Clark and Clark scale (8). Not only did the leukocytes stick to the endothelium, they also stuck to each other,

forming clumps. As such clumps increased in size, they frequently broke away from the vessel wall and were carried along in the circulation as emboli. When two such emboli came in contact they stuck together, forming larger clumps which frequently plugged the venules. Such emboli were quite soft and pliable, and when they became stuck in the venules, red blood cells and blood platelets could be seen being forced in between the leukocytes. Once a venule was plugged in this way, other emboli entering the venule came in contact with and were stopped by the obstruction. In this way the plug increased in size until the entire venule was filled.

Leukocytes also stuck to the walls of the arterioles, forming masses which interfered with the blood flow. The arterioles remained constricted to approximately one-half of their control diameters during the period of increased leukocyte sticking and this also had the effect of reducing the blood flow.

The stickiness of the endothelium began to decrease approximately two hours after the injection, and the leukocytes were gradually released from the walls of the arterioles. Most of the leukocytic plugs that had blocked the venules gradually slipped along until they reached larger vessels and were carried away in the circulation within $2\frac{1}{2}$ to $3\frac{1}{2}$ hours after the injection.

DISCUSSION. Previous studies in the literature have demonstrated that ergonovine causes vasoconstriction (9, 10, 11), but there are no reports in which the constricting vessels were actually observed microscopically and measured. The results secured by Brown and Dale (13) are in accord with the present observation that the arterioles become unresponsive following repeated injections of sufficiently large amounts of ergonovine maleate. The fact that the smaller arterioles became refractory to repeated injections before the larger ones suggests that in these experiments the cause for the development of the tachyphylaxis lay at least partly within the walls of the arterioles and arteries themselves. Thus all of the arterioles and arteries were bathed by the same blood, yet the smaller ones became unresponsive before the larger ones.

The present studies show that in amounts up to 3.0 mgm., ergonovine maleate does not cause any change in the reaction of the arterioles to epinephrine, a result which is in accord with the previous work of Davis, Adair, Chen and Swanson (9), and in marked contrast to the effect of epinephrine after ergotamine and ergotamine (12).

It is well known that gangrene can be produced only with difficulty in experimental animals using ergonovine maleate, since ergonovine is the least toxic of the ergot alkaloids, and there are no clinical reports of gangrene in man following its use. Gangrene can be produced much more readily with two other ergot alkaloids, ergotamine and ergotamine. The gangrene produced by these latter ergot alkaloids is due to obliterative endarteritis and thrombosis. Hyaline thrombi have been observed in the arteries in fixed sections (14). The formation of such thrombi is usually attributed to violent and prolonged constriction of the smaller arteries, and interruption of the blood flow, but as pointed out by Sollman (14) this is entirely hypothetical, since the process has never been ob-

served directly with the microscope in the living animal. The results secured in the present experiments following repeated injections of massive doses of ergonovine maleate suggest that such thrombi may be formed, at least in part, because of the injurious effect of ergot and its more toxic alkaloids upon the vascular endothelium, a view previously suggested on theoretical grounds by Sollman (14).

SUMMARY

1. The reaction of the arterioles, capillaries and venules to intravenous injections of ergonovine maleate as seen in transparent chambers in rabbits' ears was studied at magnifications of 200 and 400 diameters in the living animal.

2. Following initial injections of 0.005 mgm. to 4.0 mgm. of ergonovine maleate the arterioles constricted, the degree of constriction, the duration of constriction, and the effect of the blood flow being proportional, within limits, to the amount injected.

3. Injections of a given amount of ergonovine maleate made on successive days caused the same degree of arteriolar constriction each day. Repeated injections at fifteen- to thirty-minute intervals of 0.2 mgm. (twenty times the comparative clinical dose in man) or more, caused progressively less arteriolar constriction, and finally arteriolar dilatation. The smaller arterioles became refractory to repeated injections sooner than the larger ones.

4. Injections of ergonovine maleate in amounts up to 3.0 mgm. produced no change in the response of the arterioles to 0.025 mgm. of epinephrine.

5. Ergonovine maleate caused constriction of the venules when injected in amounts of 0.01 mgm. or more.

6. No active capillary constriction was seen after any of the injections.

7. Repeated injections of 0.01 mgm. (an amount comparable by weight in the rabbit to the clinical dose of 0.2 mgm. in man) made on the same and on successive days did not alter the vascular endothelium in any way detectable with the microscope. Repeated injections of massive amounts (3.0 mgm. in the rabbit, which is 300 times the comparative clinical dose in man) caused an increase in stickiness of the vascular endothelium toward leukocytes, and the formation of leukocytic masses which interfered with the blood flow and stopped it in many of the vessels. This effect was reversible, and disappeared within approximately three hours.

8. The present studies demonstrate that in amounts comparable to those used clinically, ergonovine maleate is entirely without injurious effect upon the blood vessels in the rabbit, in so far as this can be determined by direct observation with the microscope. There is a very wide margin of safety between the usual clinical dose and one which does produce toxic effects. Even when such effects were produced by massive injections, they were reversible.

The desirability of experiments such as described in this paper was first suggested to the author by Dr. K. K. Chen, and subsequently by Dr. Carl F. Schmidt, to both of whom the author is indebted.

REFERENCES

1. SMITH, R. G.: J. A. M. A., **111**: 2201, 1938.
2. DALE, H.: Schweiz. med. Wehnschr., **65**: 885, 1935.
3. DUDLEY, H. W., AND MOIR, C.: Brit. Med. J., **1**: 520, 1935.
4. MOIR, C.: Brit. Med. J., **2**: 799, 1936.
5. ADAIR, F. L., DAVIS, M. E., KHARASCH, M. S., AND LEGAULT, R. R.: Am. J. Obst. & Gynec., **30**: 466, 1935; **30**: 740, 1935.
6. ABELL, R. G., AND CLARK, E. R.: Anat. Rec., **53**: 121, 1932.
7. ABELL, R. G.: Anat. Rec., **69**: 11, 1937.
8. CLARK, E. R., AND CLARK, E. L.: Am. J. Anat., **57**: 385, 1935.
9. DAVIS, M. E., ADAIR, F. L., CHEN, K. K., AND SWANSON, E. E.: THIS JOURNAL, **54**: 398, 1935.
10. DAVIS, M. E., ADAIR, F. L., AND PEARL, S.: J. A. M. A., **107**: 261, 1936.
11. ROTHLIN, E.: Schweiz. med. Wehnschr., **65**: 947, 1935.
12. WILSON, H. C.: THIS JOURNAL, **56**: 97, 1936.
13. BROWN, G. L., AND DALE, H.: Proc. Roy. Soc., London S. B., **118**: 446, 1935.
14. SOLLMAN, T. A.: Manual of Pharmacology. 6th Edition, 1942. W. B. Saunders and Company.

NITRITES

XI. NITRIC ESTERS OF ALKYL GLYCOLLATES¹

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In a previous report (1) two of us (J. C. K., Jr., and C. J. C.) described the depressor response of the dog to intravenous injections of nitric esters of certain alkyl glycollates and of sodium glycollate. It was shown that the salt is far less potent than the alkyl esters. The former is very soluble in water, while the latter are only sparingly soluble and have high oil/water coefficients. Large doses of the salt (50 mgm. per kgm.) are required in order to produce depressor effects. Refractoriness of the animals after recovery from the first doses was reported. However, animals which were refractory to subsequent large doses of the salt were not tolerant to the alkyl esters. These esters were sufficiently potent to cause repeated deep depressor responses.

The original group of four alkyl esters of glycollate nitrate has now been extended to include all normal chain alkyl homologs from methyl to decyl, and certain branched chain isomers. It is the purpose of this study to examine the relationships between potency and water solubility and between potency and structure in this series.

METHODS. Relative depressor potency among the alkyl glycollate nitrates was determined by means of an assay in which each homolog, in 0.01 *M* hydroalcoholic solution, was compared with a solution of a standard, the concentration of which was adjusted to an appropriate value. Alternating intravenous injections of equal volumes of the unknown and of the standard were made in etherized dogs. Three or more depressor responses to each agent which were statistically equivalent constituted an assay. The standard used was the isobutyl ester of glycollate nitrate. It was found to be among the most potent of the group. The ratios of the molarities of standard solutions to those of the other members of the group in which they were comparable are termed the "isobutyl ratings" of the compounds. These ratings are an expression of relative potency. Solubility in water of all compounds was determined and related to their potency.

RESULTS. Table 1 lists the compounds prepared and studied, with the isobutyl ratings of those which were assayed, and with solubility data on all. The data in the table which concern straight chain esters are shown graphically in figure 1. The latter reveals the general relationship between potency and solubility among those members of the group. Potency increases as water solubility decreases, until the octyl compound is reached. The concentration of alcohol required for solution of the latter at 0.01 *M* strength was great (58 per cent) and renders the validity of assays of higher homologs uncertain.

¹ The expense of this investigation has been defrayed in part by a grant from Eli Lilly and Company, Indianapolis, Indiana.

TABLE 1

Relative potency and water solubility among alkyl esters of the nitric esters of glycollic acid

ALKYL ESTER 0.01 M	EQUIVALENT MOLARITY OF THE ISOBUTYL STANDARD	ISOBUTYL RATING†	WATER SOLUBILITY AT 30°C. GRAMS PER 100 CC.
Methyl.....	0.0006	0.06 ± 0.01	2.444
Ethyl.....	0.002	0.2 ± 0.05	0.291
Isopropyl.....	0.004	0.4 ± 0.05	0.176
n-Propyl.....	0.004	0.4 ± 0.05	0.181
(Isobutyl).....	0.010	1.0	0.131
sec-Butyl.....	0.003	0.3 ± 0.02	0.118
n-Butyl.....	0.004	0.4 ± 0.02	0.135
Isoamyl.....	0.010	1.0 ± 0.1	0.092
n-Amyl.....	0.0065	0.65 ± 0.05	0.099
n-Hexyl.....	0.006	0.6 ± 0.04	0.124
n-Heptyl.....	0.010	1.0 ± 0.1	0.095
n-Octyl.....	0.0065	0.65 ± 0.1	0.103
n-Nonyl.....	*	*	0.111
n-Decyl.....	*	*	0.115

* The higher members require too great a concentration of alcohol for solution at 0.01 M. Assays of potency by this method are not valid.

† The standard errors of the assays were expressed in per cent. When the assay value was converted to its "Isobutyl Rating" the standard errors were expressed in per cent of these respective ratings.

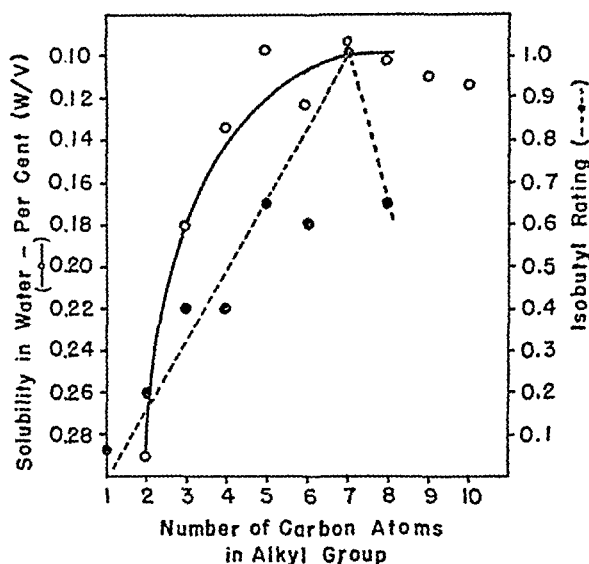


FIG. 1. Relationship between Potency and Water Solubility Among Normal Chain Alkyl Glycolate Nitrates.

It may be noted in the table that the isoamyl and isobutyl esters are much more potent than their isomers. Varying solubility data among the butyl and amyl isomers do not explain differences in their relative potency.

Discussion. The importance of oil/water coefficients in drug action has been appreciated for many decades. It is more striking in some classes of drugs than in others. It is very marked in the case of inorganic and organic nitrites, and in that of organic nitrates. In this series of compounds water solubility and oil solubility were in inverse ratio to each other (1). Among the straight chain alkyl esters of the nitrates of glycollic acid, the peak of potency was found to reside in the n-heptyl compound. This may mean that its oil/water coefficient is optimum, and that the assay method does not serve to evaluate the higher members because of the inordinately high alcoholic content necessary for their solution. Decreasing water solubility thus becomes a limiting factor in depressor potency in the series.

The high potency of the isobutyl and isoamyl esters suggests a structural factor in the determination of potency, for solubility in water does not explain it.

SUMMARY

The homologous series of alkyl esters of the nitric esters of glycollic acid has been extended to include all straight chain compounds from the methyl to the decyl and certain of the branched chain isomers. Water solubility of all, and relative depressor potency established by bioassay of all but the nonyl and decyl esters, are reported. The isobutyl ester possesses much greater potency than its normal and secondary isomers, and also more than all normal chain homologs tested with the exception of the n-heptyl compound. The relationship between water solubility and potency is presented.

REFERENCE

1. KRANTZ, J. C., JR., CARR, C. J., FORMAN, S. E., AND CONE, N.: *THIS JOURNAL*, 70: 323, 1940.

RESISTANCE TO TOXIC THIOUREAS IN RATS TREATED WITH ANTI-THYROID COMPOUNDS

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The acute toxic effects of certain thioureas in rats have been studied extensively by Richter and his collaborators (1-3). Substances such as phenylthiourea and α -naphthylthiourea (ANTU) are lethal in relatively low doses, producing massive pulmonary edema and pleural effusion. Less toxic compounds such as thiourea and several other mono-substituted derivatives also cause similar changes in rats (4). Marked differences in species susceptibility exist for these poisons, and in general herbivores are more resistant than carnivores or omnivores (3, 5). The high susceptibility of the rat, and the fact that it will readily eat amounts which are lethal has led to the extensive use of ANTU as a rodenticide.

Resistance to a toxic thiourea can be readily induced in the rat by pre-treatment with sub-lethal doses of the compound, as was first shown for phenylthiourea by Richter and Clisby (1). The same development of resistance has been reported for ANTU (2). Animals so treated can shortly tolerate doses up to 100 times that lethal to control animals. The resistance appears within 24 hours and may last for more than sixteen days (6). Most workers have described the resistance which follows pre-treatment with the same toxic compound but in one report pre-treatment with thiouracil was shown to confer protection against the toxic action of thiourea (7). It has also been shown that treatment with potassium iodide will induce resistance to ANTU in normal but not in thyroidectomized rats (8).

In the experiments to be described a study has been made of the resistance to toxic thioureas. It will be shown that the ability of any compound to protect the rat against phenylthiourea or ANTU is apparently a specific property and is not directly related to the acute toxicity or anti-thyroid activity of the particular substance.

METHODS. Adult male or female rats, of different strains, weighing over 200 gm., were maintained on Purina Fox Chow, and water *ad lib*. No significant variation in susceptibility to phenylthiourea or ANTU was noted in the different strains of rats, in the different sexes or in rats fed other "chow" diets of similar composition.

Substances were administered in water or suspended in 10 per cent gum acacia, the concentrations being adjusted so that whenever possible each dose was less than 1 cc. Acute toxicity was determined for a single dose of the substance administered intraperitoneally. Mortality was noted for the following 48 hours.

The ability of a compound to impart resistance was determined by injecting it subcutaneously on three alternate days. Eight hours after the last injection the animal received an intraperitoneal injection of 30 mgm./kgm. of body weight of phenylthiourea or ANTU—representing at least six times the normal lethal dose. This method was used in obtaining the results reported in tables 1 and 2. In an alternative assay the test substance was injected every day for three days and the animal tested with the toxic compound on the fourth day. Similarly a single injection was found to impart in 24 hours resistance to doses of 20 mgm./kgm. or more of the toxic compounds.

Colorimetric estimation of the excretion of phenylthiourea in the urine was made with Grote's reagent. This was prepared and a standard curve obtained as described by Williams, Jandorf and Kay for thiouracil (9). This method was found to be satisfactory when applied to rat's urine. An Evelyn colorimeter with 620 filter was used for the colorimetric measurements.

TABLE 1

Protective ability, acute toxicity and anti-thyroid activity of compounds which protected rats from poisoning by phenylthiourea and ANTU

COMPOUND	SOURCE	LD ₅₀	NUMBER OF RATS USED	PROTECTING DOSE ¹	NUMBER OF RATS USED	ANTI-THYROID ACTIVITY ²
		mgm./kgm.		mgm./kgm.		
Thiourea	A	10-1000 ¹	15	0.1-0.5 (0.3-1.0) ³	8 (9) ³	0.12
Phenylthiourea	EK	5	12	0.5-0.6	10	—
Allylthiourea	EK	500	11	0.1-0.8	11	—
α -Naphthylthiourea (ANTU)	D	5	8	0.5-1.0	8	—
2-Mercaptothiazoline	A	500	3	1.0-2.0 (3.0-7.0)	10 (9)	1.3
Acetylthiourea	EK	400	6	3.0-5.0	10	—
Thiothymine	S	1000	9	5.0-7.0 (10-20)	8 (9)	0.7
6-n-Propylthiouracil	L	400	8	10-15	10	11
Di-n-butylthiourea	EK	300	7	10-15	9	—
2-Thiouracil	AC	1500	6	30-45 (80-120)	30(16)	1
Thiobarbital	A	175	6	90-100	10	1.7
Potassium iodide	M	—	—	90-120+	10	—
Ammonium thiocyanate	M	—	—	100-200	7	—
5,5-Diphenyl-2-thiohydantoin	A	—	—	100-200	4	0.5

¹ Protecting Dose.—The approximate quantity which, when given at each of three injections according to the procedure described, will just protect rats from a subsequent dose of 30 mgm./kgm. of phenylthiourea. At the smaller figure, the majority of rats died, while at the larger, the majority survived.

² The figures in brackets give the results of experiments in which ANTU was used as the toxic compound rather than phenylthiourea.

³ Anti-thyroid Activity.—Relative activities based on thiouracil = 1. The figures are taken from a paper by Astwood, Bissell and Hughes (10). Although quantitative measurements are not available for some of these compounds, all of them have been shown to have anti-thyroid activity.

⁴ The toxicity of thiourea appeared to vary tremendously, a fact which has been noted previously (3).

A—Abbott, EK—Eastman Kodak, D—Dupont, S—Schwartz Laboratories, New York, L—Lederle, AC—American Cyanamid, M—Merck.

RESULTS. Acute Toxicity of Thiourea and Related Compounds. The approximate dose necessary to cause 100 per cent mortality in rats has been determined for the compounds studied. Since for the purpose of these experiments only rough comparative values were necessary the number of animals used was insufficient to establish accurate toxicity levels. The values obtained have been listed in table 1.

It may be seen that an extreme range of toxic dose levels was shown by various compounds. Whereas the more toxic substances such as ANTU and phenylthiourea required doses of only 5 mgm./kgm. to cause death, others such as allylthiourea and thiothymine required 800 to 1000 mgm./kgm.

Protective Action of Thiourea and Related Compounds. The minimal amount of the various compounds which when administered to rats would then protect them against six times the lethal dose of phenylthiourea was determined. These values are also listed in table 1, and the substances are arranged so that the more active ones are placed first.

It may be noted that the most effective substances were thiourea, phenylthiourea, and allylthiourea. When one compares the acute toxicity of the various substances with their ability to induce protection it is obvious from the results listed in the table that there is no direct relationship. Allylthiourea, for example, is a highly effective protective compound and yet is relatively non-toxic.

The protective action against ANTU has also been determined for a number of substances. These results were similar to those obtained for phenylthiourea although more of each compound was required to produce a comparable protective effect.

A number of compounds, some closely related chemically to those found to be active, have failed to confer protection against phenylthiourea in the doses tested. These substances have been given to groups of two or three rats only but failed to show any evidence of protective action. They are listed in table 2.

Anti-thyroid activity in thiourea and related compounds. For the sake of comparison the anti-thyroid activity in rats of the various substances listed in table 1 has been included. These values were not determined but were taken from a paper by Astwood, Bissell and Hughes (10). The figures shown are expressed in terms of the activity of 2-thiouracil which was given the arbitrary value of 1.

From the anti-thyroid activity of the compounds listed it can be seen that there is no direct relationship between this property and that of imparting resistance. Furthermore the acute toxicity, protective action, and anti-thyroid action appear to be non-related properties and have independent actions.

Mechanism of protective action. a) Protection in thyroidectomized animals. Six rats were thyroidectomized and two to three weeks later received protective doses of either phenylthiourea or 2-thiouracil. All animals survived the test dose of 30 mgm./kgm. of phenylthiourea. Completeness of the removal of thyroid tissue was verified by failure of the animals to survive at a temperature of 5°C. for more than five days.

b) Excretion of phenylthiourea. Excretion studies of phenylthiourea were conducted on urine of resistant rats. Four rats were treated with phenylthiourea until they showed no evidence of lung damage following a single intraperitoneal injection of 100 mgm./kgm. After this injection their urine was collected for a period of 55 hours, and specimens were treated with Grote's reagent and assayed colorimetrically. Of the 98 mgm. administered, 11.5 mgm. were excreted in the first 24 hours and 4.3 mgm. in the following 24-hour period. Amounts of this urine calculated to contain doses of 3 to 4 mgm./kgm. were given intraperitone-

ally to normal rats. These animals either died or when killed showed the lung edema and pleural effusion, typical of poisoning by thiourea compounds. Control rats injected with equivalent amounts of normal urine showed no such effects.

c) Protection against lung irritant gases. Ten rats received daily doses of thiourea until they had become highly resistant. Through collaboration of Dr. S. J. Patrick of the Defense Research Chemical Laboratories at Ottawa, they were then exposed to two toxic concentrations of phosgene.

TABLE 2
Compounds which failed to protect rats against poisoning by phenylthiourea

COMPOUND	SOURCE	DOSAGE MGM./KGM. OF BODY WEIGHT AT EACH INJECTION	COMPOUND	SOURCE	DOSAGE MGM./KGM. OF BODY WEIGHT AT EACH INJECTION
Cystine..	M	100	Methyl(1-methyl-butyl)iminothio-barbituric acid	A	200
Methionine.. . . .	M	100	Mono(1-methyl-butyl)iminothio-barbituric acid	A	200
Sulfaguanidine.....	L	200	Sodium pentobarbital	A	70
p-Aminobenzoic acid....	BDH	200	Histamine dihydrochloride	H	200
Acetonitrile	EK	200	Diethanol sulfide	EK	200
Phenylisothiocyanate ¹ .	EK	100	Urethane	BDH	200
Thiocarbanilide ²	EK	200	Calcium chloride	M	100
Monobenzylthiobarbituric acid.....	A	200	Sodium sulfate	M	200
Mono(1-methyl-butyl)-thiobarbituric acid...	A	200	Sodium thiosulfate	M	200
Allyl(1-methyl-butyl)iminothiobarbituric acid.....	A	200	Ammonium chloride	Mall	200

¹ Dissolved in propylene glycol for injection.

² Not well absorbed.

M—Merek, L—Lederle, BDH—British Drug Houses, EK—Eastman Kodak, A—Abbott, H—Hoffman-La Roche, Mall—Mallinckrodt.

Whereas all of the rats in a control group died at the higher and one of five at the lower concentration from typical phosgene poisoning, all of the treated group succumbed to the same two concentrations of gas. Their lungs showed typical edema and there was no evidence that the 'protective treatment' had increased their resistance to phosgene.

Similarly a group of rats made resistant to phenylthiourea showed no evidence of resistance when exposed to lethal concentrations of the gas carboxy sulphide (COS) or of hydrogen sulphide.

d) Species variation. Widely different toxic dose levels of ANTU for different animal species have been reported (5). Although highly toxic for rats

this substance is relatively well tolerated by rabbits. One compound investigated, thiothymine, on the other hand was particularly toxic for rabbits. Doses of approximately 100 mgm./kgm. in rabbits caused death from pulmonary edema and pleural effusion. In the guinea pig, rat and mouse, however, doses of 1000 mgm./kgm. were not always fatal and did not always cause lung edema. Splenectomy which has been described as increasing the tolerance of rabbits to thiothymine (11), did not in a few experiments affect the lethal dose level.

Attempts to demonstrate a protective substance in the blood of animals made resistant by pre-treatment with thiourea compounds were unsuccessful.

DISCUSSION. The acute toxicity and the effective dose which will protect rats against lethal doses of toxic thioureas have been determined for a number of compounds. No direct relationship between those actions could be demonstrated and compounds of low toxicity such as allylthiourea may be highly active in their protective action. The anti-thyroid activity was apparently unrelated to either of the above actions and such compounds as sulfaguanidine, p-aminobenzoic acid and acetonitrile, representing different classes of anti-thyroid drugs, had no protective action against phenylthiourea or ANTU. With the exception of potassium iodide and ammonium thiocyanate all the substances exhibiting a protective action contained the full or partial (e.g., 2-mercaptothiazoline) thiourea grouping. The protective effect was shown against ANTU as well as against phenylthiourea although somewhat larger doses were required with the former substance.

The mechanism of the protective action is not obvious. This action is apparently limited to substances of the groups described since lung edema caused by agents such as phosgene is not prevented. Protective compounds have to be administered prophylactically. If given at the same time or following the toxic substance they do not induce protection, even though animals normally do not die for 8 to 24 hours following the injection. Although the anti-thyroid potency of the compounds was not directly related to their ability to impart resistance to the lethal action, only compounds reported to have anti-thyroid activity possessed this property. The presence of a full or partial thiourea group seems to be important but not essential for the development of resistance. The development of resistance, however, is not dependent on the thyroid gland since it was found to occur readily after suitable treatment of thyroidectomized rats.

Selye has described the adaptation of animals to specific toxins by a reaction which involves an increased activity of the adrenal cortex (12). Adapted animals can resist large doses of the specific toxic compound with which they have been treated, but are more sensitive to other toxins. During this reaction there is an associated adrenal hypertrophy. Such a mechanism would not seem related to the resistance described for thiourea-like substances since this resistant state is not specific for any individual compound but applies to many related substances. Furthermore, it is not related to the toxicity of the compound and animals treated with anti-thyroid substances tend to show an atrophy rather than hypertrophy of the adrenal glands.

It seemed possible that the resistant animal might have developed an ability to detoxify compounds which were usually lethal. Excretion studies, however,

indicated that appreciable amounts of phenylthiourea were excreted in the urine of resistant rats after administration of a large dose. The injections of such urine was toxic to normal animals. These results indicate that resistant rats do not entirely detoxify phenylthiourea and their resistance must therefore be explained by some other mechanism.

The species variation in the toxicity of the various compounds was striking and no obvious explanation for this phenomenon was found. It is of interest that ergothioneine, a thiourea-like substance, has been isolated from red blood corpuscles (13), but the amounts of this substance present in the blood of different species cannot be correlated to their susceptibility to ANTU (14). Although herbivorous animals appear less affected by most substances, the high susceptibility of the rabbit to thiothymine is an exception. Furthermore, in this case the guinea pig, although eating the same diet as the rabbit, is relatively resistant. The relationship of diet to protection against thiourea compounds will be described in a subsequent paper.

SUMMARY

1. The ability of thiourea-like substances to confer protection against the highly toxic members of this series, was found to be unrelated to the acute toxicity, or anti-thyroid activity of the compound.

2. The rat which is made resistant apparently has the ability to resist the lung edema and pleural effusion which is found in untreated animals killed by lethal doses of toxic thioureas. Such resistance is developed only to drugs of this type since no protection against irritant gases such as phosgene or hydrogen sulphide was detected.

3. The thyroidectomized rat may be made resistant as readily as the intact animal.

4. Following the administration of a large dose, phenylthiourea is excreted in the urine by the resistant rat in quantities toxic to normal animals.

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REFERENCES

1. RICHTER, C. P., AND CLISBY, K. H.: *Arch. Path.*, **33**: 46, 1942.
2. RICHTER, C. P.: *J. A. M. A.*, **129**: 927, 1915.
3. RICHTER, C. P.: *Recent Progress in Hormone Research*, **2**: 255, 1948. New York, Academic Press.
4. DIEKE, S. H., ALLEN, G. S., AND RICHTER, C. P.: *THIS JOURNAL*, **90**: 260, 1947.
5. DIEKE, S. H., AND RICHTER, C. P.: *Proc. Soc. Exper. Biol. & Med.*, **62**: 22, 1946.
6. RICHTER, C. P.: *Proc. Soc. Exper. Biol. & Med.*, **63**: 364, 1946.
7. LANDGREBE, F. W., AND MORGAN, T. N.: *Nature, London*, **157**: 22, 1946.
8. BYERRUM, R. U., AND DU BOIS, K. P.: *THIS JOURNAL*, **90**: 321, 1947.
9. WILLIAMS, R. H., JANDORF, B. J., AND KAY, G. A.: *J. Lab. & Clin. Med.*, **29**: 329, 1944.
10. ASTWOOD, E. B., BISSELL, A., AND HUGHES, A. M.: *Endocrinol.*, **37**: 456, 1915.
11. CULLUMBE, H., AND SIMPSON, M.: *Nature, London*, **159**: 781, 1947.
12. SELYE, H.: *J. Clin. Endocrinol.*, **6**: 117, 1946.
13. WILLIAMSON, S. W., AND MELDRUM, N. U.: *Biochem. J.*, **26**: 815, 1932.
14. HUNTER, G.: *Biochem. J.*, **22**: 4, 1928.

THE ANTIMALARIAL ACTIVITY OF 2, 4-DIAMINO-6, 7-DIPHENYL PTERIN; ITS POTENTIATION BY SULFADIAZINE AND INHIBITION BY PTEROYLGLUTAMIC ACID

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During the course of routine screening of compounds for antimalarial activity against *Plasmodium gallinaceum* in the chick, a group of synthetic pterins were tested. Daniel *et al.* (1) had shown that certain diaminopterins inhibited the growth of *Streptococcus faecalis* and *Lactobacillus casei* which require preformed pteroylglutamic acid, and *L. arabinosus* which synthesizes its own. These investigators (2) found that 2-amino-4-hydroxy pterins inhibited growth and hemoglobin formation in chicks. Dr. C. K. Cain, whose group synthesized these pterins (3, 4), supplied us with the following compounds:

DR 15,789 2,4-Diamino-6,7-dimethylpyrimido (4,5-b) pyrazine

DR 15,790 2,4-Diamino-6,7-dicarboxypyrimido (4,5-b) pyrazine

DR 15,791 2,4-Diamino-6,7-diphenylpyrimido (4,5-d) pyrazine

DR 15,793 2-Amino-4-hydroxy-6,7-dimethylpyrimido (4,5-b) pyrazine

DR 15,792 2-Amino-4-hydroxy-6,7-diphenylpyrimido (4,5-b) pyrazine

DR 15,794 2,4-Diamino-6,7-bis (p-aminophenyl)-pyrimido (4,5-b) pyrazine

METHODS. *Test for antimalarial activity.* The methods employed have been previously described (5). Week-old New Hampshire red chicks, weighing between 42 and 52 gm., were used throughout. The chicks were inoculated intravenously with 16×10^6 erythrocytes parasitized with *Plasmodium gallinaceum*. Treatment was begun four to five hours before the inoculation of parasites and continued twice daily for four days. The drugs were administered orally in gelatin capsules. On the morning following the last dose of drug, parasitemia was determined as the number of parasitized erythrocytes per 10^4 erythrocytes. The minimum effective dose of drug was that amount which caused a 75 per cent reduction in mean parasitemia in the five treated chicks as compared with the ten untreated controls.

Toxicity in the chick. The subacute toxicity of the compounds was determined by Dr. Nathan B. Eddy, to whom thanks are extended. The chicks were treated in the same manner as in the therapeutic tests. The maximum tolerated dose was the largest dose which allowed any gain in weight.

EXPERIMENTAL. In table 1 are given the toxicity for the chick and the antimalarial activity of each of the compounds tested. Only one compound, DR 15,791, the 2,4-diamino-6,7-diphenyl pterin, showed any antimalarial activity at doses tolerated by the chick.

Since these compounds were reported to be antagonists of pteroylglutamic acid (1) the effect of this metabolite on the antimalarial activity of DR 15,791 was studied. The chicks were maintained on a stock diet of commercial chick mash (Purina Startena), the pteroylglutamic acid content of which was not

known to us. Synthetic pteroylglutamic acid was administered in the same manner as the pterin, twice daily in gelatin capsules. Results of one such experiment are shown in table 2. When pteroylglutamic acid was administered at the rate of 0.02 mgm./gm. twice daily (approximately 2 mgm. per chick per day) there was a significant but not complete reversal of antimalarial activity of the pterin while the inhibition resulting from pteroylglutamic acid at 0.2 mgm./gm. twice daily (20 mgm. per chick per day) was slightly greater. In earlier experiments

TABLE 1

The subacute toxicity for chicks and the activity of certain synthetic pterins against Plasmodium gallinaceum

COMPOUND	MAXIMUM TOLERATED DOSE. MGM./GM. TWICE DAILY FOR 4 DAYS*	MINIMUM EFFECTIVE DOSE FOR ANTIMALARIAL ACTIVITY MGM./GM. TWICE DAILY FOR 4 DAYS†
DR 15,793	0.3	>0.3
DR 15,791	0.25	0.016
DR 15,790	>0.5	>0.5
DR 15,792	0.4	>0.4
DR 15,794	>0.5	>0.5
DR 15,789	0.05	>0.05

* Greatest amount which 3 chicks survived with a final weight (on the morning after the last dose) at least equal to the starting weight.

† Lowest dose which caused a 75 per cent reduction in mean parasitemia in 5 treated chicks as compared with 10 untreated controls.

TABLE 2

The inhibition of the antimalarial activity of diamino, diphenyl pterin by pteroylglutamic acid against Plasmodium gallinaceum in the chick

DOSEAGE OF PTERIN MG./GM. b.i.d.	DOSEAGE OF PTEROYLGLUTAMIC ACID MG./GM. b.i.d.	4TH DAY PARASITE COUNT (PARASITIZED ERYTHROCYTES/10 ⁴ ERYTHROCYTES)
Untreated		7330
0.02		339
0.02*	0.02	2816
0.02	0.2	5030

* 10 chicks used in this group.

it had been found that pteroylglutamic acid at 0.002 mgm./gm. caused no significant reversal of the antimalarial activity of DR 15,791.

Daniel and Norris (6) have shown that the synthetic pterins potentiated the activity of sulfonamides against bacteria which synthesize their own pteroylglutamic acid. When DR 15,791 was administered at one-fourth minimum effective dose (0.0039 mgm./gm.) it required about one-sixteenth the minimum effective dose of sulfadiazine to obtain an antimalarial response (table 3). When the pterin was administered at one-half minimum effective dose, it required less than one thirty-second the minimum effective dose of sulfadiazine to produce

antimalarial activity. As seen in table 4, DR 15,739 and 15,794, but not DR 15,790, were able, at maximum tolerated doses, to be activated by sub-effective doses of sulfadiazine.

DISCUSSION. Of six synthetic pterins studied only one, 2,4-diamino-6,7-diphenyl pterin (DR 15,791) had any antimalarial activity in doses which were tolerated by the chick though the 6,7-dimethyl and dianiline analogues were active when administered with sub-effective doses of sulfadiazine. The antimalarial activity of DR 15,791 was of about the same order as quinine when tested against

TABLE 3

The potentiation of the antimalarial activity of diamino, diphenyl pterin by sulfadiazine against Plasmodium gallinaceum in the chick

DOSAGE OF PTERIN MGM./GM. TWICE DAILY FOR 4 DAYS	4TH DAY PARASITEMIA (PARASITIZED ERYTHROCYTES/10 ⁴ ERYTHROCYTES) DOSAGE OF SULFADIAZINE (MGM./GM. TWICE DAILY FOR 4 DAYS)*						
	None	0.00094	0.001875	0.00375	0.0075	0.015	0.03
None	7570				6560	3834	750
0.0039	7580	2876	1157	25	93	7	
0.0078	5640	363	3	2	4	2	
0.0156	1280						

* Bold-faced type indicates mean parasite counts which are at least 75 per cent below the mean parasite count of the corresponding untreated controls.

TABLE 4

The potentiation of the antimalarial activity of 2,4-diamino pterins by sulfadiazine

DOSAGE OF PTERIN MGM./GM. TWICE DAILY FOR 4 DAYS	4TH DAY PARASITEMIA (PARASITIZED ERYTHROCYTES/10 ⁴ ERYTHROCYTES) DOSAGE OF SULFADIAZINE (MGM./GM. TWICE DAILY FOR 4 DAYS)*					
	None	0.0019	0.0038	0.0075	0.015	0.03
None	7270			4780	1852	184
DR 15,790—0.5	7360	6420	6240	4520		
DR 15,789—0.05	6800	3452	1636	92		
DR 15,794—0.5	7220	3310	3360	1066		

* Bold-faced type indicates mean parasite counts which are at least 75 per cent below the mean parasite count of the corresponding untreated controls.

P. gallinaceum (5). Since most of the important antimalarials are many times more active than quinine, against *P. gallinaceum*, this synthetic pterin is not particularly promising for the therapy of human malaria.

However, the compound behaves in many ways identically with chlorguanide (Paludrine), a widely used antimalarial. Chlorguanide has been found to potentiate sulfadiazine in its activity against *P. gallinaceum* to approximately the same degree as the diamino, diphenyl pterin (7). Furthermore, chlorguanide activity is significantly but incompletely inhibited by approximately the same amounts of pteroylglutamic acid as are required to inhibit the pterin (8). None

of the antimalarial compounds tested other than chlorguanide, p-aminobenzoic acid competitors and the diamino, diphenyl pterin, can be inhibited by pteroylglutamic acid. Furthermore, other commonly used antimalarials, such as quinine, chloroquine, or pamaquin, have been found unable to potentiate sulfadiazine (9). The resemblance in behavior between chlorguanide and the diamino, diphenyl pterin is, therefore, striking and we have concluded that the mode of action of the two drugs is similar.

In comparing the structural formulae of the pterin and chlorguanide it will be noted that both compounds contain a biguanido configuration, which in the pterin is condensed into a ring. Hawking and Perry (10) have shown that chlorguanide is inactive *in vitro* against exoerythrocytic forms of *P. gallinaceum*. Passage of the drug through the chick or incubating it with chick liver apparently converts chlorguanide to a substance which has antimalarial activity. Preliminary results indicate that the pterin is not active *in vitro* against erythrocytic parasites. It is possible that both these compounds are changed in the body to produce similar moieties with antimalarial activity.

SUMMARY

A series of six pterins were tested for antimalarial activity against *Plasmodium gallinaceum* in the chick. Of these only one, the 2,4-diamino-6,7-diphenylpyrimido (4,5-b) pyrazine (DR 15,791), was able to suppress parasitemia at doses tolerated by the chick. The antimalarial activity of DR 15,791 was about the same as that of quinine. It was markedly potentiated by sulfadiazine in its activity against *P. gallinaceum* and it was significantly, but not completely, inhibited by pteroylglutamic acid *in vivo*. In these respects and in the fact that it contains a biguanide linkage, it resembles the antimalarial chlorguanide (Paludrine). The 6,7-dimethyl and dianiline, but not the 6,7-dicarboxylic acid, analogues of DR 15,791 were active as antimalarials when administered with sub-effective doses of sulfadiazine.

REFERENCES

1. DANIEL, L. J., NORRIS, L. C., SCOTT, M. L., AND HEUSER, G. F.: J. Biol. Chem., 169: 689, 1947.
2. DANIEL, L. J., SCOTT, M. L., NORRIS, L. C., AND HEUSER, G. F.: J. Biol. Chem., 173: 123, 1948.
3. CAIN, C. K., MALLETTE, M. F., AND TAYLOR, E. C., JR.: J. Am. Chem. Soc., 68: 1996, 1946.
4. MALLETTE, M. F., TAYLOR, E. C., JR., AND CAIN, C. K.: J. Am. Chem. Soc., 69: 1814, 1947.
5. COATNEY, G. R., AND SEBRELL, W. H., in WISELOGLE, F. Y.: A Survey of Antimalarial Drugs, 1941-1945. J. W. Edwards, Ann Arbor, Michigan, 1946.
6. DANIEL, L. J., AND NORRIS, L. C.: J. Biol. Chem., 170: 747, 1947.
7. GREENBERG, J., BOYD, B. L., AND JOSEPHSON, E. S.: THIS JOURNAL, 94: 60, 1948.
8. GREENBERG, J.: Proc. Soc. Exper. Biol. and Med., 71: 306, 1949.
9. GREENBERG, J.: unpublished.
10. HAWKING, F., AND PERRY, W. L. M.: Brit. J. Pharm., 3: 320, 1948.

THE MECHANISM OF TOLERANCE TO THIOPENTAL IN MICE

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Numerous studies on the development of tolerance to barbiturates have been reported. In only two of these studies was any attempt made to study the mechanism involved. Masuda, Budde, and Dille (1) observed a slightly increased rate of disappearance of Amytal from the blood, muscle, and liver, of tolerant animals. Green and Koppányi (2) found no difference between the rates of excretion of barbital in tolerant and control animals and concluded that the tolerance was probably due to "cellular or histogenic phenomena." The development by Goldbaum (3,4) of a sensitive method for the determination of barbiturates in biological materials makes possible the reinvestigation of this problem.

EXPERIMENTAL. *Effect of fourteen daily thiopental injections on sleeping time and tissue barbiturate levels at awakening.* The criteria of wakefulness used were those described by Winter (5). The animals were classified as "awake" on the basis of the following criteria—ability to walk in normal fashion, eyes wide open, fur not erect, nose and vibrissae in characteristic motion, and efforts to escape when picked up. Sixty male mice weighing 25 to 27 gm. were randomized into two equal groups. On the first day of the experiment each animal was given thiopental, 50 mgm./kgm. i.p. and its sleeping time observed. Thereafter the experimental group was given 50 mgm./kgm. of thiopental i.p. in 0.2 cc. of solution daily for thirteen days, while the control group received 0.2 cc. of saline i.p. On the fourteenth day of the experiment both groups of animals were given thiopental 50 mgm./kgm. and their sleeping times again recorded. As the animals awakened they were bled by decapitation and placed in groups of five in order of time of awakening. Equal samples of blood, brain, liver and kidney from each animal in the group were pooled to make composite samples of each tissue. The thiopental concentrations in each of these tissues and the remaining carcass were determined. The concentration of thiopental in the total body was calculated from the amount of barbiturate in the carcass plus the amount in the blood and tissues removed.

Effect of seven daily thiopental injections on sleeping time and tissue barbiturate levels at awakening. A similar experiment was carried out without a preliminary determination of sleeping time to avoid giving thiopental to the control group. Fifteen experimental mice received daily i.p. injections of thiopental 50 mgm./kgm. and the control mice received daily saline injections for six days. On the seventh day both groups were given thiopental 50 mgm./kgm. and the sleeping times recorded. At awakening, the animals were sacrificed for analysis as described above.

Daily changes in sleeping time and tissue levels of thiopental at awakening. Fifty male mice weighing 19 to 20 gm. were randomized into ten groups of five. The mice in each of the groups were given a daily intraperitoneal injection of thiopental 50 mgm./kgm., and their sleeping times recorded. On each day of the experiment, the mice in one randomly selected group of animals were bled and sacrificed on awakening, and the plasma and tissue barbiturate concentrations determined as above. Thus, the animal sacrificed on day 1 received

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only one injection of barbiturate; those sacrificed on day 5 received five injections; and so on to the tenth day. The mean sleeping time of each group on the first day of the experiment served as the control sleeping time for that particular group; the sleeping times on subsequent days were expressed as per cent of initial sleeping time.

TABLE 1

Comparison of sleeping times and tissue levels at awakening in 30 mice receiving 14 daily i.p. injections of thiopental 50 mgm./kgm. with those of 30 saline-injected controls

SLEEPING TIME	CONTROL	EXPERIMENTAL	SIGNIFICANCE LEVEL
	Geometric mean \pm S.E.	Geometric mean \pm S.E.	P =
Original	40 \pm 5 min.	39 \pm 3.1 min.	—
After two weeks.....	43 \pm 7 min.	20 \pm 2.1 min.	<.005
TISSUE LEVELS OF THIOPENTAL AT AWAKENING	MEAN \pm S.D.	MEAN \pm S.D.	
Brain.....	18 \pm 0.9 μ gm./gm.	21.4 \pm 2.7 μ gm./gm.	<.05
Kidney.....	23.4 \pm 1.5 μ gm./gm.	30.5 \pm 3.1 μ gm./gm.	<.001
Liver.....	54.0 \pm 6.5 μ gm./gm.	81.3 \pm 12.2 μ gm./gm.	<.01
Plasma.....	24.6 \pm 3.3 μ gm./cc.	29.5 \pm 1.62 μ gm./cc.	<.02
Total Body.....	.030 \pm .006 mgm./gm.	.040 \pm .006 mgm./gm.	<.05

TABLE 2

Comparison of sleeping times and tissue levels at awakening in 15 mice receiving 7 daily i.p. injections of thiopental 50 mgm./kgm. with those of 15 saline-injected controls

Sleeping time	CONTROL	EXPERIMENTAL	SIGNIFICANCE LEVEL
	Geometric mean \pm S.E.	Geometric mean \pm S.E.	P =
	43 \pm 6 min.	22 \pm 2 min.	<.005
Tissue levels of thiopental at awakening	Mean \pm S.D.	Mean \pm S.D.	
Brain	16.2 \pm 1.0 μ gm./gm.	20.7 \pm .65 μ gm./gm.	<.01
Kidney.....	33.9 \pm 11.2 μ gm./gm.	43.9 \pm 11.2 μ gm./gm.	>.10
Liver.....	49.2 \pm 3.1 μ gm./gm.	73.4 \pm 8.2 μ gm./gm.	<.02
Plasma.....	22.6 \pm .6 μ gm./cc.	28.0 \pm 1.6 μ gm./cc.	.02
Total Body.....	.032 \pm .005 mgm./gm.	.043 \pm .008 mgm./gm.	>.10

RESULTS AND DISCUSSION. As shown in table 1, there was a significant reduction of sleeping time in the animals receiving a daily dose of thiopental as compared with the saline-injected controls at the end of fourteen days. The experimental animals awakened at significantly higher tissue levels than did the controls. The results of the seven-day study are shown in table 2. They are essentially the same as those obtained in the fourteen-day study. The differences between control and experimental thiopental concentrations in kidney and total

body, although of the same magnitude as those in the first experiment, were not statistically significant. This may have been due to the smaller number of animals and tissue analyses (fifteen control and fifteen experimental animals which permitted only six barbiturate determinations on each tissue).

The daily changes in sleeping time and thiopental tissue levels at awakening are shown in figure 1. The mean sleeping times diminished progressively to the fifth day after which there was no further significant regression. The levels of thiopental in the tissues and in the total body at awakening showed a progres-

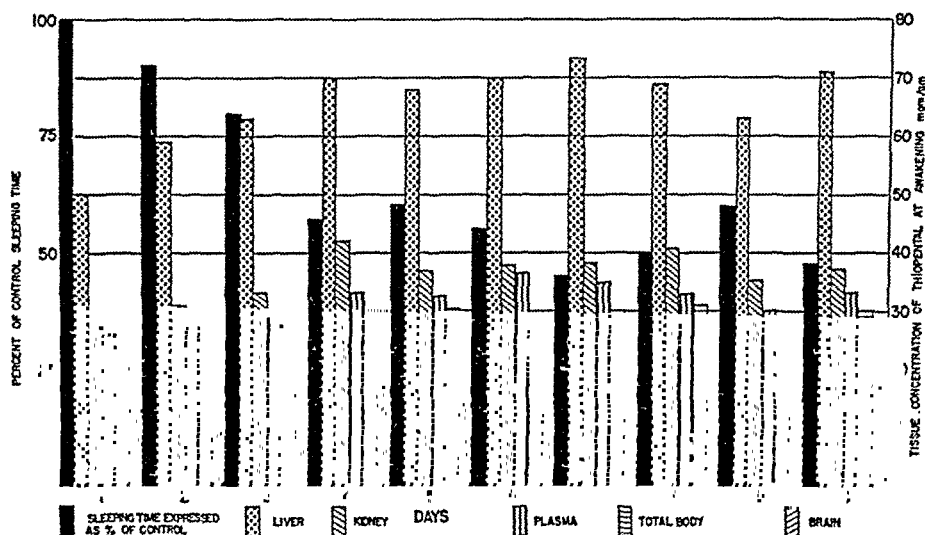


FIG. 1. The relation between number of days injected with sleeping time and awakening thiopental tissue levels in mice receiving daily i.p. injections of thiopental 50 mgm./kgm.

TABLE 3

The correlation coefficients between tissue levels of thiopental at awakening and per cent reduction of sleeping time

	TISSUE				
	BRAIN	PLASMA	KIDNEY	LIVER	TOTAL BODY
Coefficient of correlation					
± S.E.	.88 ± .33	.92 ± .33	.82 ± .33	.84 ± .33	.80 ± .33
P	<.001	<.001	<.01	.001	<.01

sive increase to the fifth day with no significant change with further daily injections. Thus as the animals' tissue levels at awakening increased, their sleeping times diminished accordingly. In table 3 are shown the coefficients of correlation between tissue levels at awakening and per cent reduction of sleeping time. These correlations are statistically significant.

The results of these experiments indicate that mice develop a tolerance to thiopental, and with the dose used the tolerance is maximal in five to six days.

The higher total body levels in the adapted animal rules out the possibility of an increased rate of destruction or excretion. The tolerance mechanism appears to be an adaptation to higher thiopental tissue levels.

SUMMARY

1. Mice develop a tolerance to the daily administration of 50 mgm./kgm. of thiopental. This tolerance is maximal in five to six days and amounts to a decrease in sleeping time of about 50 per cent.

2. The tolerant animals awaken at higher tissue levels than controls. The degree of tolerance as determined by per cent reduction in sleeping time is significantly correlated with the tissue levels at awakening.

3. The tolerance mechanism appears to be one of adaptation to higher thiopental tissue levels and not to either an increased rate of excretion or destruction of thiopental.

REFERENCES

1. MINORU, M., BUDDE, R. N., AND DILLE, J. M.: *Jour. Am. Pharm. A.*, 27: 830, 1938.
2. GREEN, M. W., AND KOPFANYI, T.: *Anesthesiol.*, 5: 329, 1944.
3. JAILER, O. W., AND GOLDBAUM, L. R.: *J. Lab. and Clin. Med.*, 31: 1344, 1946.
4. GOLDBAUM, L. R.: *THIS JOURNAL*, 94: 68, 1948.
5. WINTER, C. A.: *THIS JOURNAL*, 94: 7, 1948.

CARDIO-RESPIRATORY EFFECTS FOLLOWING INTRAVENOUS ADMINISTRATION OF SOME ANTIHISTAMINIC DRUGS

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Despite the considerable increase in the clinical use of antihistaminics and also the chemical similarity they bear to certain cardiotoxic or antifibrillatory compounds such as procaine, a survey of the literature reveals that scant attention has been paid to the direct or indirect action of these drugs on the heart. Pyranisamine (Neoantergan) has been reported to exert a quinidine-like action on isolated auricular tissue from rabbits (1). In normal persons, McGavack, Elias and Boyd (2) found that diphenhydramine (Benadryl) administered orally in maximal therapeutic doses for several weeks, seldom lowered systolic blood pressure and did not produce detectable alterations in the heart. Winder and Thomas (3), experimenting on dogs, found no gross change in rhythm primarily attributable to the latter compound. On the isolated rabbit heart, they noted that Benadryl produced a slight to moderate decrease in the amplitude of systolic contraction. Scudi and Reinhard (4) studied the action of thonzylamine (Neohetramine). They attributed its hypotensive effect on the cat to cardiac dilatation, and also noted a diminished systolic contraction of both the frog's and turtle's heart.

Criep and Aaron (5) mention T wave changes in their investigation of patients with heart disease treated with phenindamine (Thephorin). Mackmull (6), studying patients receiving Benadryl intravenously, reported changes in the amplitude of the QRS, T, and P waves; the P-R interval was prolonged in one case after 300 mgm. The effects were ascribed to a direct action on the myocardium.

More extensive studies have been performed on the effects of antihistaminic drugs on blood pressure and respiration. They are well reviewed by Loew (7) and Haley (8).

METHODS. In the present study, the effects of some antihistaminic drugs on the mammalian heart were investigated using the cat as experimental animal and noting particularly changes in the electrocardiogram derived from the three standard leads. Blood pressure (mercury manometer) and respiration (pneumatic bag around the chest) were also recorded simultaneously for supplementary information. The following compounds² were

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² Chemical names in the order listed are as follows: 2-(N-benzyl-N-phenyl-aminomethyl) imidazoline hydrochloride (Antistine Hydrochloride); β -Dimethylaminoethyl benzhydryl ether hydrochloride (Benadryl Hydrochloride); N,N-Dimethyl-N'-(α pyridyl) ethylene diamine monohydrochloride (Pyribenzamine Hydrochloride); N,N-Dimethyl-N'-(α -pyridyl)-N'-(α -thenyl)-ethylenediamine hydrochloride (Thenylene Hydrochloride); 2-Methyl-9-phenyl-2,3,4,9-tetrahydro-1-pyridindene hydrogen tartrate (Thephorin).

tested²: phenazoline (Antistine Hydrochloride); diphenhydramine (Benadryl Hydrochloride); tripeleminamine (Pyribenzamine Hydrochloride); methapyrilene (Thenylene Hydrochloride); phenindamine (Thephorin).

The drugs, one per cat, were administered intravenously in concentrations of 4-5 mgm./cc. at 15-minute intervals in gradually increasing doses until the lethal dose was reached. Five to eight cats per drug were used. They were anesthetized with "Dial" 0.7 cc. per kgm. intraperitoneally.

The results are summarized in table 1. They exhibit a definite consistency, not only in the individual experiments with a single drug, but also in the groups of experiments with different compounds. Therefore, we may discuss the effects of the drugs generally rather than specifically. One mgm./kgm. of any one of the drugs produced practically no respiratory or ECG change and only slight hypotension followed by a small but prolonged rise (fig. 1, A and B; fig. 2, A, B and C). The 4 and 8 mgm./kgm. doses uniformly produced transient arterial hypotension, a brief period of apnea followed by a short interval of tachypnea, and a considerable decrease in heart rate (fig. 1, A and B). The magnitude of the effects was well correlated with the dose. The changes were always reversible after the 4 mgm./kgm. dose, and usually after the 8 mgm./kgm. dose also except for two fatalities out of 29 cats tested, produced by the latter dose, one with Antistine, and the other with Benadryl. The period required for recovery of the blood pressure was generally less than 1½ minutes (fig. 1, A) but occasionally the effects were more prolonged (fig. 1, C and D).

The electrocardiogram showed significant changes after both the 4 and 8 mgm./kgm. doses, the effects of the latter being similar but more marked. The most important electrocardiographic changes observed were an increase of the P-R interval and a widening of the QRS complex. These effects were consistent after the 4 mgm./kgm. doses but with the number of experiments involved the differences were not large enough to be statistically significant until after the 8 mgm./kgm. injections. The largest increases of both the P-R and QRS intervals were produced by Thephorin; Thenylene, Antistine, Pyribenzamine and Benadryl came next in decreasing order (figs. 2 and 3). As illustrated in the figures, the ventricular complexes, especially after the 8 mgm./kgm. dose, often showed the bundle branch block configuration (fig. 2, Column F; fig. 3, bottom row).

Another very consistent electrocardiographic change produced by the 4 and 8 mgm./kgm. doses was a decreased amplitude of the R wave with a concomitant increase of the amplitude of the S wave in Leads II and III (figs. 2 and 3). This result was statistically significant for all the drugs after the 8 mgm./kgm. dose. Variable effects were always noted on the T wave also but there was no consistency, either for the specific drugs or for the group as a whole. In some cases where it could be determined, the Q-T interval was found to increase under the influence of the antihistaminic agents tested.

The reviewed changes were well correlated with the dose injected and were

² The drugs were supplied through the courtesy of the following: Antistine and Pyribenzamine from Ciba Pharmaceutical Products, Inc., Benadryl from Parke, Davis & Co., Thenylene from Abbott Laboratories and Thephorin from Hoffmann La Roche, Inc.

TABLE I

		BLOOD PRESSURE	HEART RATE	P-R Sec.	QRS Sec.	R mm.	S mm.	T mm.
Antistine (5)	Before treatment	104 ± 16	169 ± 16	.090 ± .007	.034 ± .004	3.9 ± 0.7	1.4	0.25
	1 mgm./kgm.	82 ± 15	152 ± 28	.087 ± .0037	.035 ± .003	2.8 ± 0.6	1.0 ± 0.7	0.8 ± 0.5
	4 mgm./kgm.	37 ± 5	68 ± 6	Nodal	.057 ± .010	3.3 ± 0.5	3.5 ± 0.9	1.1 ± 0.4
	8 mgm./kgm.	30 ± 4	103 ± 18	Rhythm .107 ± .0056	.080 ± .020	2.0 ± 0.9	3.5 ± 0.7	1.4 ± 0.7
Benadryl (6)	Before treatment	106 ± 8	185 ± 11	.070 ± .004	.033 ± .002	5.7 ± 0.6	2.1 ± 1.0	1.1 ± 0.8
	1 mgm./kgm.	104 ± 8	178 ± 11	.071 ± .003	.033 ± .002	5.1 ± 0.5	2.6 ± 0.7	1.2 ± 0.5
	4 mgm./kgm.	68 ± 3	163 ± 12	.078 ± .004	.040 ± .005	4.1 ± 0.4	3.8 ± 0.9	1.3 ± 0.6
	8 mgm./kgm.	29 ± 10	146 ± 11	.090 ± .006	.065 ± .008	3.4 ± 0.6	5.3 ± 1.0	1.7 ± 0.5
Pyribenzamine (8)	Before treatment	123 ± 13	180 ± 11	.080 ± .004	.036 ± .002	4.5 ± 0.8	2.0 ± 0.9	1.5 ± 0.3
	1 mgm./kgm.	114 ± 13	167 ± 13	.083 ± .005	.040 ± .004	3.1 ± 0.6	1.3 ± 0.7	0.6 ± 0.2
	4 mgm./kgm.	48 ± 6	142 ± 13	.089 ± .003	.050 ± .004	1.7 ± 0.3	2.3 ± 0.6	0.5 ± 0.2
	8 mgm./kgm.	27 ± 3	135 ± 9	.101 ± .006	.075 ± .008	1.2 ± 0.3	3.4 ± 0.6	1.1 ± 0.2
Thenylene (6)	Before treatment	115 ± 10	166 ± 20	.080 ± .0035	.038 ± .002	4.7 ± 0.7	1.4 ± 1.1	0.8 ± 0.5
	1 mgm./kgm.	110 ± 10	159 ± 17	.080 ± .007	.038 ± .002	3.6 ± 0.75	2.4 ± 1.2	1.5 ± 0.6
	4 mgm./kgm.	73 ± 14	140 ± 8	.094 ± .009	.060 ± .007	2.4 ± 0.6	3.5 ± 1.5	1.1 ± 0.3
	8 mgm./kgm.	55 ± 9	139 ± 9	.114 ± .013	.086 ± .017	1.7 ± 0.8	4.5 ± 2.0	0.5 ± 0.6
Thephorin (5)	Before treatment	93 ± 8	175 ± 20	.075 ± .014	.034 ± .003	3.9 ± 1.3	1.5 ± 1.1	0.5 ± 0.5
	1 mgm./kgm.	73 ± 9	155 ± 18	.078 ± .011	.040 ± .006	3.1 ± 0.8	2.5 ± 1.6	0.9 ± 0.4
	4 mgm./kgm.	43 ± 9	143 ± 22	.094 ± .010	.068 ± .011	2.4 ± 1.1	6.0 ± 3.1	2.6 ± 0.7
	8 mgm./kgm.	40 ± 7	133 ± 23	.144 ± .022	.100 ± .012	1.0 ± 1.1	4.8 ± 1.5	1.9 ± 0.5

Numerals in parentheses following drug names indicate number of cat experiments for each drug.
 Figures indicate averages and their standard error.

fleeing in nature. Always after the 4 mgm./kgm. dose and also after the 8 mgm./kgm. dose with only two exceptions (previously mentioned), the electrocardiograms recorded fifteen minutes after the injection appeared very much the same as the controls recorded before injection (fig. 2, Col. G). Fig. 2, row 2, Column G,

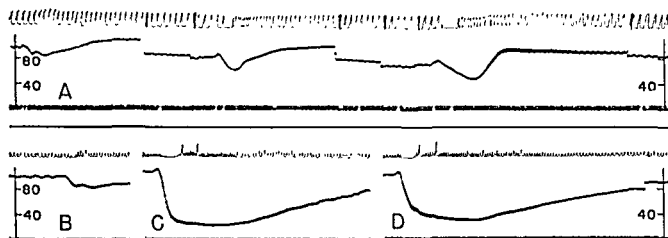


FIG. 1. Typical blood pressure and respiratory changes with 1, 4 and 8 mgm./kgm. of Thephorin (A) and Antistine (B, C, and D).

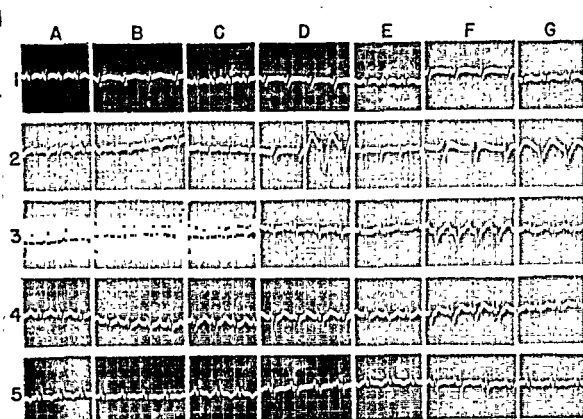


FIG. 2. 1, Pyribenzamine; 2, Antistine; 3, Thephorin; 4, Benadryl; 5, Thenylene. A, Normal; B, Maximum effect after 1 mgm./kgm. injection; C, recovery; D, after 4 mgm./kgm.; E, recovery; F, after 8 mgm./kgm.; G, recovery.

illustrates one of the exceptions, the fatality produced by the 8 mgm./kgm. dose of Antistine. A rather peculiar effect of Antistine, especially following the 4 mgm./kgm. dose, was to produce nodal rhythm, considerably slower than the previous S-A rhythm.

Beyond the 8 mgm./kgm. dose, the toxic effects of the drugs were very pronounced, and included marked hypotension, prolonged apnea, occasionally followed by Cheyne-Stokes respiration (fig. 6) and severe bradycardia. Death gen-

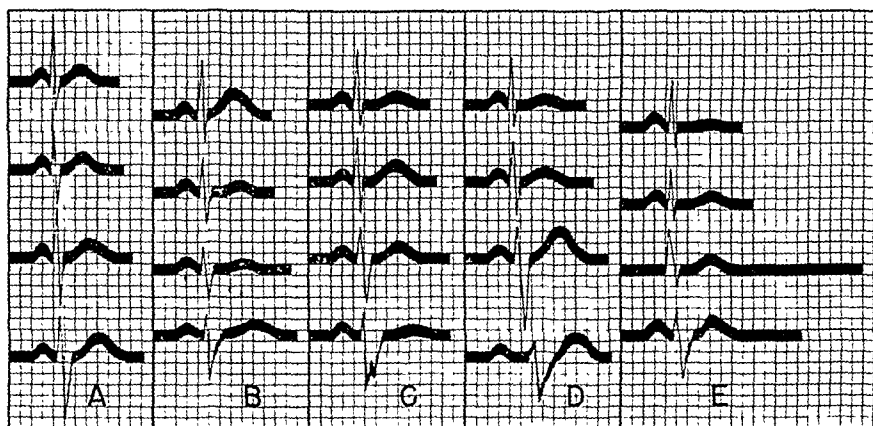


FIG. 3. Average maximum effects of 1, 4 and 8 mgm./kgm. doses in descending order, the top row representing normals. A, Benadryl; B, Pyribenzamine; C, Thenylene; D, Thephorin; E, Antistine.

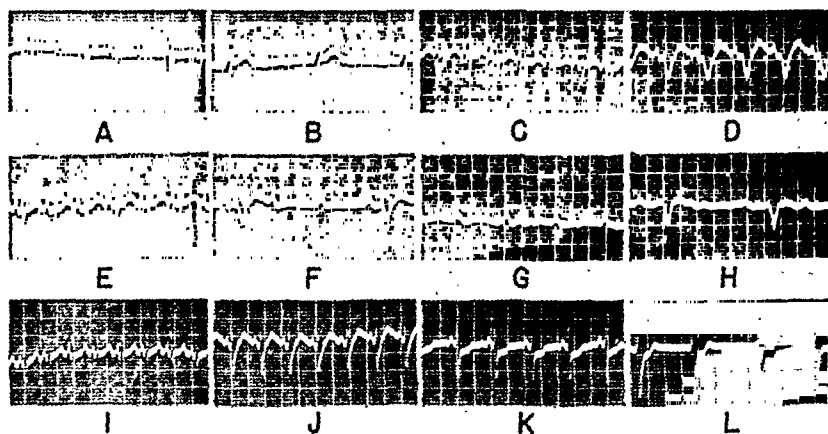


FIG. 4. A, Normal ECG; B, after 2nd dose (5 mgm./kgm.) of Antistine; C, recovery; D, after 4th dose (10 mgm./kgm.); E, recovery; F, after 5th dose (15 mgm./kgm.); G, one minute after F; H, Terminal, about $3\frac{1}{2}$ to 4 minutes after F; I, Normal ECG; J, after 8 mgm./kgm. dose of Benadryl; K, few minutes later showing partial recovery from drug effects but anoxia; L, Terminal.

erally resulted from doses ranging between 12 and 20 mgm./kgm. of any of the substances tested. The electrocardiographic changes following these highly toxic injections indicated profound changes in the properties of the heart muscle and conducting tissue (fig. 4 and 5).

Severe degrees of partial A-V block were common; there were occasional in-

stances of complete A-V block, sometimes with permanent ventricular standstill; there were also examples of marked intraventricular conduction defects, coupled beats, and nodal rhythm. Isolated premature contractions were never seen. On some occasions depressed S-T segments typical of anoxia were observed coincidentally with cessation of respiration (fig. 4, K and L).

Death following lethal doses of the antihistaminics tested was due either to respiratory paralysis, to circulatory failure or to a combination of both. No con-

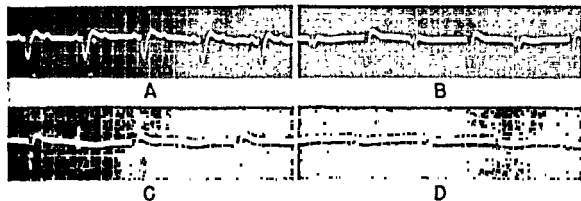


FIG. 5. A, B, C, D—Progression of terminal effects with fatal dose of Pyribenzamine

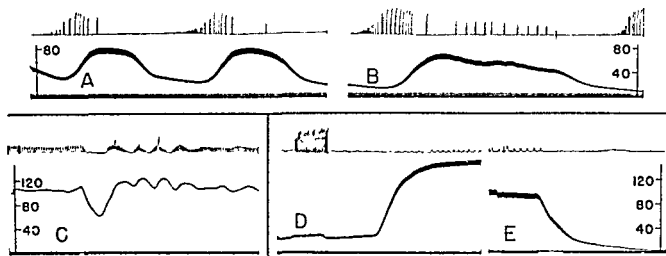


FIG. 6. A and B, Cheyne-Stokes respiration produced by 16 mgm./kgm. of Thephorin showing relationship of maintenance of blood pressure with respiration; C, another example of Cheyne-Stokes with Thephorin at 4 mgm./kgm.; D, effects of epinephrine after artificial respiration failed following 16 mgm./kgm. dose of Thienylene; E, 20 mgm./kgm. were fatal and epinephrine had no effect.

sistent trend could be elicited from the data in this regard with any of the tested drugs.

Discussion. Most of the antihistaminics possess the trialkylamino group which is in common with other drugs having local anesthetic, anti-allergic and quinine-like properties. These investigations confirm the suspected cardiac effects of the antihistaminics tested, which consisted principally of a depressant action on conduction.

It must be kept in mind that the drugs were administered intravenously and in much larger doses than in common clinical usage. However, in view of pre-

vious findings already quoted, together with the results of these experiments, one might suggest some caution at least in administering large doses of antihistaminics to patients who also suffer from heart disease.

SUMMARY

To ascertain their effects on blood pressure, electrocardiogram, and respiration, the following drugs were intravenously administered to cats anesthetized with intraperitoneal Dial: Antistine, Benadryl, Pyribenzamine, Thenylene and Thephorin.

With 1 mgm./kgm. of any one of the drugs, there was practically no respiratory or ECG change and only a slight hypotension followed occasionally by a small but prolonged rise. The 4 and 8 mgm./kgm. doses produced reversible diminutions of blood pressure, and apnea for a few seconds followed by brief tachypnea. The electrocardiograms showed auriculo-ventricular block of the 1st degree, intraventricular block, slowing of the rate and changes in electrical axis. The effects were well correlated with the dose.

After the 8 mgm./kgm. dose, results included marked hypotension, various arrhythmias, and exaggeration of the previous ECG and respiratory effects. Death was due either to respiratory paralysis, cardiac failure, or both. The antihistaminics tested have consistent cardiac and respiratory depressant effects when given intravenously to cats in the doses mentioned.

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REFERENCES

1. DEWS, P., AND GRAHAM, J. D. P.: *Brit. J. Pharmacol.*, **1**: 278, 1946.
2. MCGAVACK, T. H., ELIAS, H., AND BOYD, L. J.: *J. Lab. and Clin. Med.*, **31**: 560, 1946.
3. WINDER, C. V., AND THOMAS, R. W.: *THIS JOURNAL*, **91**: 1, 1947.
4. SCUDI, J. V., REINHARD, J. F., AND DREYER, N. B.: *J. Allergy*, **19**: 184, 1948.
5. CRIEF, L. H., AND AARON, T. H.: *J. Allergy*, **19**: 215, 1948; **19**: 304, 1948.
6. MACKMULL, G.: *J. Allergy*, **19**: 365, 1948.
7. LOEW, E. R.: *Physiol. Rev.*, **27**: 542, 1947.
8. HALEY, T. J.: *J. Am. Pharm. A. (Scient. Ed.)*, **37**: 383, 1948.

THE EFFECT OF BOTULINUS TOXIN ON THE ELECTROMYOGRAM

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Studies on the nature of the muscular weakness of botulism have all shown that an important part of the effect of Botulinus toxin is due to its action on the peripheral parts of the neuro-muscular system. Edmunds and Long (1) and Bishop and Bronfenbrenner (2) found that the muscle of animals paralyzed by the toxin would respond to direct stimulation but not to stimulation of the motor nerve. The latter investigators also showed, by a study of action potentials of nerve, that conduction in nerve trunks is unimpaired. The conclusion has therefore been reached, on the basis of these and other experiments, that the peripheral effect of the toxin is an impairment of neuro-muscular conduction similar to that produced by curare. More recently, reports by Torda and Wolff (3) and by Guyton and McDonald (4) have indicated that an important feature of the paralysis resulting from Botulinus toxin is a failure of production of acetylcholine from nervous tissue, and that botulism is by no means identical with curarization.

The action of curare on neuro-muscular conduction is well-known and can readily be identified by a study of the action potentials of muscle. Brown (5) has described the characteristic effect as a rapid decline in the size of the muscle potential during repetitive stimulation of the motor nerve. Even a single stimulus is followed by a depression of neuro-muscular transmission which reduces the size of a second response for as long as 0.8 second.

Since muscle action potentials will disclose minor degrees of curarization, we have used this method of studying the action of Botulinus toxin and have observed the effect of anti-curarizing agents upon the neuro-muscular failure.

METHOD. The initial experiments were performed on guinea pigs, as these animals are particularly susceptible to the effect of Botulinus toxin. When the toxin was injected subcutaneously or intraperitoneally in doses from one-half to one MLD, symptoms usually appeared in from two to four days. The first sign was often a change in behavior. In some instances, the animal appeared hyper-irritable and would rush wildly about the cage if disturbed. Later, it usually became quiet and drowsy. Respiratory symptoms developed early and consisted of slow, labored breathing with prolonged expiration. Shortly after this, the animal became weak. The weakness developed first in the hind legs, which seemed to drag as the animal tried to run. Later, weakness was so severe that all movements were embarrassed. In severely affected animals, there was frequently observed a fine twitching of the whiskers, suggesting irritative phenomena as well as paralytic ones. Several animals were lost because of the short period between the onset of weakness in the legs and death.

For this reason, experiments were usually started before severe respiratory embarrassment had occurred. The animals were sedated with small divided doses of sodium pentobarbital because they tolerated sedation less well than the normal animal. An intravenous cannula was inserted to permit intravenous injection, and all medication was given by this route.

The tendon of the anterior tibial muscle was dissected free below the transverse tarsal ligament, and then released at its distal end by removing a small wedge of bone from around the point of insertion. The foot was amputated, permitting the tendon to be led straight down from the muscle without interference from surrounding tissue. The leg was then fixed firmly in a myograph clamp by passing a small drill through the head of the femur and one through the malleoli. The distal end of the tendon was fastened rigidly by a clamp. A pair of chlorided silver stimulating electrodes were applied to the sciatic nerve, and the action potentials of the anterior tibial muscle were recorded through chlorided silver needles thrust into the belly and the tendon of the muscle. The belly lead was grounded. Stimulation was by means of a thyratron stimulator capable of frequencies up to 90/sec. Supramaximal stimuli were used throughout. Potentials were amplified by a condenser coupled amplifier, with time constant 1.0 sec., and recorded from a Matthews oscillograph.

Muscle action potentials were studied at different stages of muscular weakness in a total of 24 animals. Seven normal animals were also examined to provide a control and to test the actions of various drugs.

In a later series of animals it was possible to avoid the systemic effects and the respiratory paralysis by inducing a "local botulism" by intramuscular injection of *Botulinus* toxin. One-tenth to one-twentieth of the minimum lethal dose of toxin injected into the anterior tibial muscle of one leg of cats or guinea pigs produced weakness in four to six days which was confined to that leg. The opposite leg was unaffected and served in these experiments as a control.¹

RESULTS. When a normal animal is studied by this technique, a simple diphasic action potential of from six to twelve millivolts is recorded. When a train of stimuli is applied, the size of the action potentials is well maintained at rates below a frequency of 40 per second. Depending upon the condition of the animal, when the frequency of stimulation reaches 40 per second there is a slight falling off in the size of the potential as stimulation continues (fig. 1a, 4c). This tendency is accentuated as the frequency is increased, but up to 60 per second, the size of the potential rarely falls to below 70 per cent of the initial response during a one-second tetanus. When pairs of stimuli are applied at varying intervals, there is usually no significant difference between the size of the two potentials recorded. Potentials separated by less than 8 milliseconds cannot be measured due to overlap.

In an animal showing muscle weakness due to *Botulinus* toxin, the size of the action potentials is smaller than normal, varying between three and six millivolts. In addition, the response to a tetanus shows a qualitative difference from normal, for during continued high frequency stimulation the size of the potential tends to increase rather than to decrease and it falls below its initial value only after prolonged stimulation at frequencies above 40 per second (fig. 1b, 4b). Under these conditions, the maximum potential may be as much as 50 per cent greater than the initial response. When pairs of stimuli are applied, the response to the second is found to be greater than that to the first (fig. 2). This potentiating effect of one response upon subsequent ones lasts up to 300 millisec., after which the two responses of a pair are again equal in size.

¹ The analogy of this state of restricted weakness to "local tetanus" is obvious. However, the analogy is quite incomplete, since muscle fibrillation, which is active in tetanus (6), could not be demonstrated in the *Botulinus*-poisoned muscle when explored with a needle electrode.

These effects of botulism were observed in animals suffering from systemic poisoning or in locally poisoned muscle. There was no difference in the action potential studies in the two.

In view of the conclusions of other investigators that the action of Botulinus toxin is similar to that of curare, several studies were made of the action of curarine on the guinea pig in order to compare the resulting abnormality with the effect of botulism. The outstanding feature of the response following this drug in the guinea pig, as in other animals, is the rapid falling off of the size of the potentials during continued stimulation (fig. 1c, 4a). This is the first observable effect of curarine, and it develops some time before paralysis occurs. The decline in the size of the potentials usually begins after the first response, but in some cases, especially with small doses of curarine, there occurs during the first three or four responses an increase in size, which is then in turn followed by rapid fall to a lower level. Study of the responses to pairs of stimuli (fig. 2) gives further evidence of early potentiation and a later depression. If the second stimulus

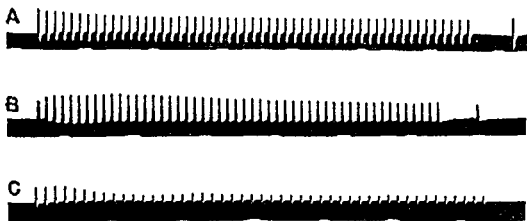


FIG. 1. Action potentials recorded from tibialis anticus during supra-maximal stimulation of sciatic nerve at 40 per second. (a) Normal guinea pig. (b) Guinea pig four days after intraperitoneal injection of one MLD Botulinus toxin. (c) Normal guinea pig after intravenous injection of 0.1 mgm. curarine. Time—1/5 second.

is set up after 70 milliseconds, its response is smaller than that to the first stimulus. This depression becomes increasingly greater for about 500 milliseconds, after which the second response gradually approaches the first in size. Some depression may be observed, however, for as long as two seconds after a single response. With large doses of curarine, the depression is more marked and develops earlier, so that potentiation is not observed. Under these conditions, the second response of a pair is always smaller than the first regardless of the interval between the stimuli. These responses after curarine are similar to those reported by Brown (5) in the cat and Harvey *et al.* (7) in the human.

The muscle action potentials of animals poisoned with Botulinus toxin show, therefore, some similarities to those obtained during curarization, but also certain differences. In each case the potentials are diminished in size and in each an early potentiation occurs during stimulation. The differences are, however, more marked than the similarities. The long-lasting depression of curarization is completely absent in botulism, and the decline in size of the action potentials which

follows the early potentiation of curarization is lacking in the records of Botulinus poisoning. For these reasons, it seems likely that the fundamental disturbance is different in the two conditions.

As a further check on this conclusion, we examined on animals with botulism the effect of drugs which are antagonistic to curare. A drug which has been shown to have a striking antagonistic effect to curare is neostigmine (Prostigmin). In an animal showing mild curare poisoning, the administration of Prostigmin causes a great increase in the size of the muscle action potential. Fig. 3b shows the electromyogram of a muscle poisoned with Botulinus toxin. After

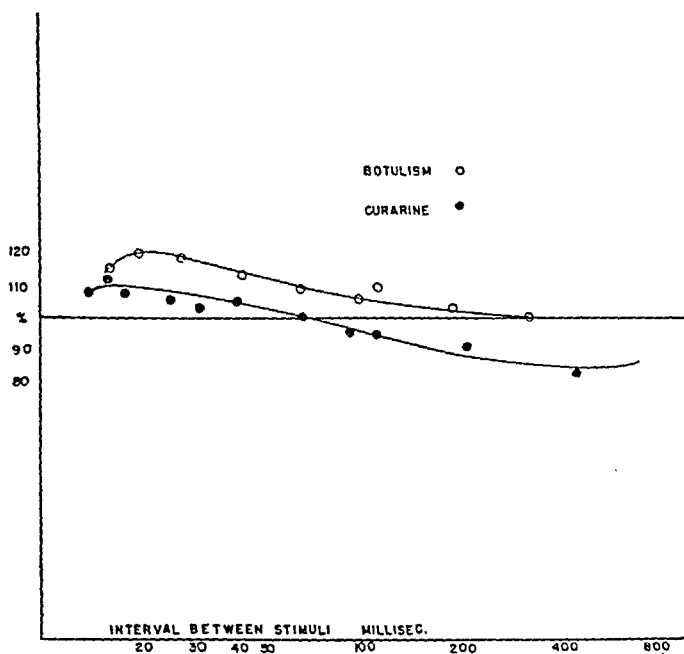


FIG. 2. Chart showing relation between size of second response (plotted as per cent of the first) and interval between stimuli applied to the nerve. Solid dots, normal guinea pig after 0.1 mgm. curarine. Circles, guinea pig four days after intraperitoneal injection of one MLD Botulinus toxin.

small doses of Prostigmin (Fig. 3c) there is a very slight increase in the size of the action potential (amounting to about 10 per cent). Larger doses produce the typical Prostigmin depression without further increasing the size of the potential. The small increase in size of the action potential following the injection of Prostigmin is insignificant in relation to the normal action potential recorded from the other leg of the same animal. These findings, so different from those observed in curarized animals, provide further evidence of a marked difference between curarization and botulism. Similar negative results were obtained with potassium chloride and with guanidine. In a single guinea pig with botulism, the intravenous injection of potassium chloride in amounts sufficient to produce car-

dine arrest failed to cause any change in the electromyogram. A similar negative result was obtained with guanidine.

The condition in which the action potentials bear the greatest resemblance to those of botulism is low calcium tetany. Brown and Harvey (8) showed that muscle potentials in animals on a low calcium diet show a progressive increase in size during repetitive stimulation. This potentiating effect lasts as long as 0.2 seconds without evidence of depression. This effect of low calcium is readily reversed by the intravenous administration of calcium salts. In order to determine whether the botulism effect can be attributed to low calcium, the effect of the administration of calcium chloride was determined. However, it was found that the intravenous injection of calcium chloride in doses up to 0.5 gm. failed

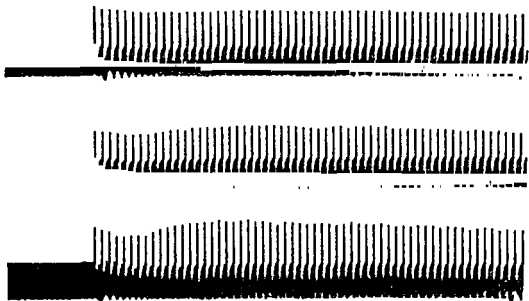


FIG. 3. Action potential recorded from tibialis anticus during supra-maximal stimulation of sciatic nerve at 40 per second. Kitten, (1 kgm.) received two MLD (guinea pig). Botulinus "B" toxin through three intramuscular injections in the right tibialis anticus four days prior to test. (a) Left (normal) leg. (b) Right (poisoned) leg. (c) Right leg ten minutes after intravenous injection of 0.1 mgm. Prostigmin and 1 mgm. atropine sulphate. Time—1.0 seconds. Calibration—10 millivolts.

to produce any change in the muscle action potential in the Botulinus-poisoned guinea pig.

These studies indicate that the paralysis in botulism differs from several conditions in which involvement of the neuro-muscular junction is a prominent feature. It, therefore, seemed desirable to re-examine the previously reported observations, that the basis of this paralysis is purely junctional.

In order to determine the extent to which actual loss of muscle contractility might be a factor in the paralysis, a comparison was made between the response to direct stimulation of a locally poisoned muscle and that of the unaffected contralateral muscle of the same animal.

For this experiment, muscle tension, as well as the electromyogram, was recorded and stimulation was either direct or indirect. The technique was similar to that previously described, except that the tendon of the anterior tibial mus-

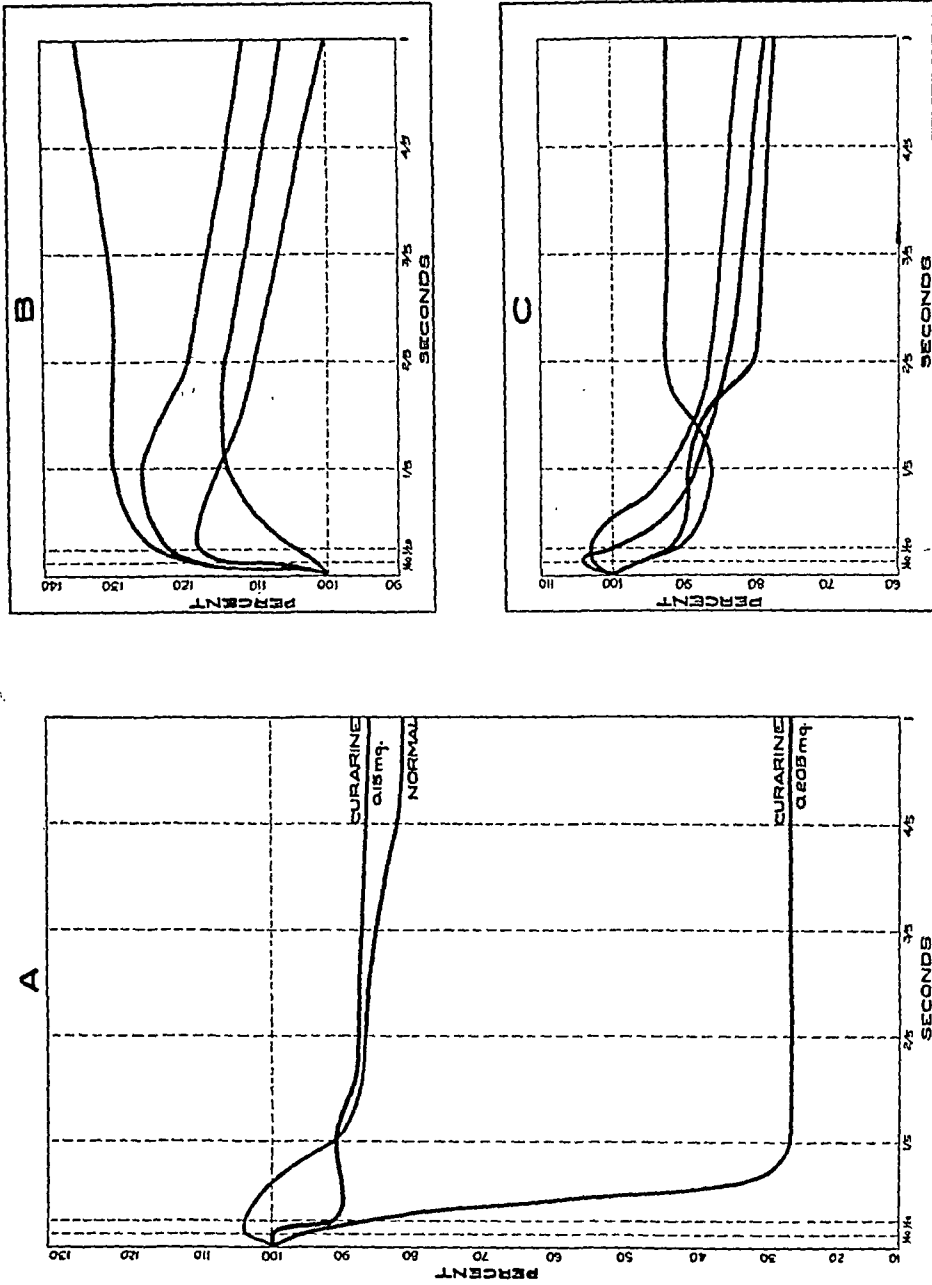


FIG. 4. Graph showing changes in size of action potential of tibialis anticus on stimulation of the sciatic nerve for one second at a frequency of 40 per second. A. Normal guinea pig before and after the intravenous injection of increasing amounts of curarine. B. Records from four different guinea pigs with various degrees of Botulinus intoxication. C. Records from four normal guinea pigs. In each instance the size of successive active potentials is plotted as per cent of the size of the first response of the tetanus.

cle was fastened to an isometric myograph lever, activating a mirror recording device. Indirect stimulation was through the sciatic nerve, as described above. Direct stimulation was carried out through the belly-to-tendon leads previously used for recording. Table 1 gives the results of such an experiment. It will be observed that the action potential and the tension developed on stimulation of the nerve were only one-fourth as great in the poisoned muscle as they were in the normal. On direct stimulation of the muscle, the contraction obtained was two-thirds of the normal. These results suggest that in addition to the neuro-muscular block there is also an actual disturbance of muscle contractility in the poisoned muscle.

DISCUSSION. The experiments cited above indicate that although a failure of neuro-muscular conduction is a feature of both botulism and curarization, there is a marked difference between the two conditions. The form of the electromyogram is different, the response to Prostigmin is different, and in botulism there is a diminution in the response to direct stimulation of the muscle which is not observed in curarization. It is evident that the action of Botulinus toxin is more

TABLE 1

Responses of normal and botulinus-poisoned muscle to direct and indirect stimulations

	TENSION GRAMS	ACTION POTENTIAL MILLIVOLTS
Normal muscle		
Indirect stimulation	2050	43.5
Direct stimulation	2050	
Botulinus-poisoned muscle		
Indirect stimulation	420	9.6
Direct stimulation	1400	

wide-spread than that of curare. The experiments were undertaken in the hope that Prostigmin might prove a useful therapeutic agent in botulism, but the results indicate that this is not the case.

Although it is clear that the electromyogram in botulism differs from that in curarization, we have not established the factors which determine this difference. It would be of interest to find out what causes in botulism the progressive increase in size of the action potential during a tetanus. Records made at high paper speed indicate that there is a change in the form of the action potential during stimulation. For this reason, it is not possible to say whether the increase in spike height is due to an increase in the number of muscle fibers responding, a change in the action potential of the individual fibers, or a change in their temporal dispersion.

SUMMARY AND CONCLUSIONS

- 1) The effect of Botulinus toxin on the electromyogram of the guinea pig and the cat has been studied.

2) The muscle action potential of a poisoned muscle is smaller than the normal. It shows a progressive increase in size during a tetanus.

3) The effect of Botulinus toxin differs from that of curare in the form of the electromyogram, the failure of Prostigmin to reverse its action, and the failure of the muscle to respond normally on direct stimulation.

4) The electromyogram observed in a Botulinus-poisoned muscle resembles that in low calcium tetany, but the effect is not reversed by intravenous injections of calcium chloride.

5) A condition of "local botulism", suggestive of "local tetanus", may be produced by the intramuscular injection of the toxin. However, the muscle is flaccid rather than rigid, and no fibrillation is observed.

6) There is no indication that Prostigmin is a useful therapeutic agent in combating muscle weakness in botulism. Potassium and guanidine are also without beneficial effect.

REFERENCES

1. EDMUNDS, C. W., AND LONG, P. H.: J. A. M. A., 81: 542, 1923.
2. BISHOP, C. H., AND BRONFENBRENNER, J. J.: Am. J. Physiol., 117: 393, 1936.
3. TORDA, C., AND WOLFF, H. G.: THIS JOURNAL, 89: 321, 1947.
4. GUYTON, A. C., AND McDONALD, M. A.: Arch. Neurol. and Psychiat., 57: 578, 1947.
5. BROWN, G. L.: J. Physiol., 92: 23P, 1938.
6. HARVEY, A. M.: J. Physiol., 96: 348, 1939.
7. HARVEY, A. M., AND MASLAND, R. L.: THIS JOURNAL, 73: 304, 1941.
8. BROWN, G. L., AND HARVEY, A. M.: J. Physiol., 97: 330, 1940.

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- Axelrod, Julius, and Brodie, Bernard B. Phenacetin: fate of, in man; methods for estimation of, and its metabolites in biological material, 58
- Beglin, Nettie, Lands, A. M., and Siegmund, O. H. Bronchodilator activity of some analogs of Isuprel, 14
- Beraldo, Wilson T., and Rocha e Silva, M. Biological assay of antihistaminics, atropine and antispasmodics on guinea pig gut, 388
- Bhattacharya, Bani K., Glasco, Elizabeth M., and Chase, Harold F. Curarizing and anti-curarizing action of group of neostigmine-like compounds, 409
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- deBeer, Edwin J., Castillo, Julio C., and Phillips, Arthur P. Curariform action of decamethylene - 1,10 - bis - trimethyl-ammonium bromide, 150
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- deEds, Floyd, and Ambrose, Anthony M. Effect of rutin and related compounds on cutaneous capillaries, 115
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- Edison, Ann O., Boxer, George E., and Jelinek, Viola C. Streptomycin: clearance and binding to protein, 93
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- Featherstone, R. M., Gross, E. G., Schueler, F. W., and Wang, S. C. Absorption spectra and pharmacological actions of tetrazole derivatives, 266
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- Fruggiero, E. J., Stutzman, J. W., and Pettinga, F. L. Cardiac effects of methoxamine and desoxyephedrine during cyclopropane anesthesia, 385
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